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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/625,100	07/22/2003	Santiago Munne		8781

7590 11/23/2005

Santiago Munne
220 Washington Str. Apt 4
Hoboken, NJ 07030

EXAMINER	
TON, THAIAN N	
ART UNIT	**PAPER NUMBER**
1632	

DATE MAILED: 11/23/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/625,100	MUNNE, SANTIAGO	
	Examiner	**Art Unit**	
	Thaian N. Ton	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) 1-4 is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) 1-4 is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☒ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date 7/22/03.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Claims 1-4 are pending and under current examination.

Claim Objections

Claims 1-3 are objected to because of the following informalities:

1. The claims do not start with a sentence "I (or we) claim, " or "The claimed invention is" (or the equivalent). See MPEP §608.01 (m).

2. Claims 1 and 2 do not end in a period.

3. Claim 3 has multiple periods (see parts a, b, and e). Each claim should begin with a capital letter and end with a period. Periods may not be used elsewhere in the claims other than for abbreviations. See MPEP §608.01 (m) and *Fressola v. Manbeck,* 36 USPQ2d 1211 (D.D.C. 1995).

4. Claim 3 sets forth a plurality of elements or steps but they are not separated by a line indentation (see also, 37 CFR 1.75 (i)). Particularly, part e) of the claim has two steps (fixing and analysis by FISH) and then Identifying and Isolating. It is suggested that the identification and isolation step be amended to be step f).

5. The claim numbering is objected to, they are not appropriately numbered as c1, c2, etc. It is suggested to number the claims as 1.; 2. etc.

Appropriate correction is required.

Information Disclosure Statement

Applicants' IDS, filed 7/22/03, has been considered.

Oath/Declaration

The oath or declaration is defective. A new oath or declaration in compliance with 37 CFR 1.67(a) identifying this application by application number and filing date is required. See MPEP §§ 602.01 and 602.02.

The oath or declaration is defective because:

The citizenship of the inventor is designated as "American". This is unclear (it encompasses North, South and Central America). If the citizenship of the inventor is from the United States, Applicants are requested to change this to "United States."

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 3-4 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for methods of producing disomic human embryonic cell lines by culturing trisomic human embryos onto mouse feeder cells, consisting of mouse embryonic fibroblast cells, wherein the mouse embryonic fibroblast cells have been previously been mitotically inactivated by mitomycin C in gelatin-tissue culture dishes, maintain said mouse feeder cells using DMEM as claimed, supplementing the medium with human LIF, culturing the embryos in said medium until day 12, fixing and analyzing said embryonic cell lines, identifying and isolating disomic cell lines within said embryonic cell lines wherein disomic cell lines are produced, does not reasonably provide enablement for the production of embryonic <u>stem</u> cell lines, or stem cell lines using the claimed methods. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims.

Enablement is considered in view of the Wands factors (MPEP 2164.01(A)). These include: nature of the invention, breadth of the claims, guidance of the specification, the existence of working examples, state of the

art, predictability of the art and the amount of experimentation necessary. All of the Wands factors have been considered with regard to the instant claims, with the most relevant factors discussed below.

Nature of the Invention/Breadth of the claims. The invention is directed to methods of producing disomic human embryonic cell lines by culturing trisomic human embryos onto mouse feeder cells, consisting of mouse embryonic fibroblast cells, wherein the mouse embryonic fibroblast cells have been previously been mitotically inactivated by mitomycin C in gelatin-tissue culture dishes, maintain said mouse feeder cells using DMEM as claimed, supplementing the medium with human LIF, culturing the embryos in said medium until day 12, fixing and analyzing said embryonic cell lines, identifying and isolating disomic cell lines within said embryonic cell lines wherein disomic cell lines are produced.

Guidance of the Specification/The Existence of Working Examples. The specification teaches chromosomally abnormal human embryos were cultured in sequential media, and the trophectoderm of the hatching blastocyst were biopsied to confirm chromosomal abnormality. The specification teaches that the remainder of the embryo was then plated on mouse embryonic fibroblast feeder layers. The embryos were then cultured until day 12, where the human cells were fixed and analyzed by FISH. A progressive increase from abnormal to normal cells was found between day 6 and day 12, and that by day 12, all 7 cultured embryos were mosaics. The specification teaches that this observed reduction in trisomic cells cannot be due to the non-survival of trisomic embryos in culture, and that the most reasonable explanation is that the trisomic cells revert to disomic cells in extended culture. See pages 3-4 of the specification and Table 1 (page 6). The specification teaches that this method can be used to obtain chromosomally normal stem cells from trisomic embryos. The specification provides a prophetic example of how to derive a

disomic cell line from these cells (see pp. 6-7, Method for derivation of single-cell clones).

State of the Art/Predictability of the Art. The breadth of the claims encompasses embryonic stem cells, which would be pluripotent and exhibit characteristics of embryonic stem cells. For example, Thomson *et al.* (**PNAS**, 92:7844-7848 (August 1995)) teach the specific, art-recognized characteristics of pluripotent cells - that these cells remain undifferentiated in culture in continuous passage, maintain a normal karyotype, express appropriate cell markers [alkaline phosphatase, SSEA-3, SSEA-4, TRA-160-, TRA-1-81] and, when injected into SCID mice, they consistently differentiate into derivatives of all three germ layers. See *Abstract* and p. 7845-7846. Although the specification provides guidance to show that embryonic cells can be produced, using the claimed methods, there is no guidance with regard to the particular markers expressed by these cells, or that these cells have differentiation potential of pluripotent cells. The specification is only directed to the karyotypic analysis of the embryonic cells (see Table 1). There is no guidance to show the isolation of inner cell mass cell from the embryos, and the subsequent analysis of the cells to show that they would indeed show the characteristics of embryonic stem cells. Indeed, the specification states that in the initial study, trophectoderm and inner cell mass cells were not independently fixed for further FISH analysis (see paragraph 13, page 4). Furthermore, the specification clearly states that the yield of disomic cells from the chromosomally abnormal embryos is extremely low, as only 7/44 embryos developed in culture until day 12. See p. 5, paragraph 18.

The Amount of Experimentation Necessary. Accordingly, in view of the state of the art of embryonic stem cells, namely the specific, art-recognized characteristics of such cells, the lack of teaching, guidance or characterization of cells produced by the claimed method, other than karytotypic analysis of the cells, the unpredictable state of the art of producing embryonic stem cells,

and the lack of guidance or teaching provided by the specification to overcome these unpredictibilities, it would have required undue experimentation for one of skill in the art to make and use the claimed disomic cell lines.

Claim Rejections - 35 USC § 102

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1 and 2 are rejected under 35 U.S.C. 102 (b) as being anticipated by Thomson [WO 96/22362, published 25 July 1996].

The claims are directed to a disomic cell line derived from trisomic embryos, and a stem cell line derived from disomic cell lines.

Note that the claims are product-by-process claims. Where, as here, the claimed and prior art products are identical or substantially identical, or are produced by identical or substantially identical processes, the PTO can require an applicant to prove that the prior art products do not necessarily or inherently possess the characteristics of his claimed product. See In re Ludtke, supra. Whether the rejection is based on "inherency" under 35 USC 102, on "prima facie obviousness" under 35 USC 103, jointly or alternatively, the burden of proof is the same, and its fairness is evidenced by the PTO's inability to manufacture products or to obtain and compare prior art products. In re Best, Bolton, and Shaw, 195 USPQ 430, 433 (CCPA 1977) citing In re Brown, 59 CCPA 1036, 459 F.2d 531, 173 USPQ 685 (1972). Further, see MPEP §2113, "Even though product-by process claims are limited by and defined by the process, determination of patentability is based on the product itself. The patentability of a product does not depend on its method of production. If the product in the product-by-process claim is the same as or obvious from a product of the prior art, the claim is unpatentable even though the prior product was made by a different process."

Thomson teach the isolation and purification of primate embryonic stem cells that are capable of indefinite proliferation *in vitro* in an undifferentiated state, are capable of differentiation to derivatives of all three embryonic germ layers, and maintain a normal karyotype throughout prolonged culture. The pluripotent cells are negative for SSEA-1, positive for the SSEA-3 marker, positive for the SSEA-4 marker, TRA-1-60, TRA-1-81 and alkaline phosphatase. Thomson teach that the primate cells can continue to proliferate in an undifferentiated state for at least one year. See p. 7, lines 9-32. Thomson teach that tumors formed after injection of rhesus ES cells into the hindleg muscles of SCID mice [see Figure 5].

Accordingly, Thomson *et al.* anticipate the claimed invention.

Claims 1 and 2 are rejected under 35 U.S.C. 102(b) as being anticipated by Shamblott *et al.* [**PNAS**, 95:13726-13731 (1998)].

The claims are directed to a disomic cell line derived from trisomic embryos, and a stem cell line derived from disomic cell lines.

The claims are product-by-process claims (see above). Shamblott teach that human pluripotent stem cells were isolated from gonadal ridges and mesenteries of 5- to 9-week postfertilization human embryos. Cells were cultured and subsequently passaged onto a mouse STO fibroblast feeder layer. Shamblott teach that embryoid bodies were collected from cultures and immediately embedded or replated into single wells [under conditions using mouse embryo fibroblasts, human fetal fibroblasts, or gelatin-coated tissue culture, see p. 13729, 1st column, 1st full ¶] and cultured for 14 days in the absence of hrLIF, hrbFGF and forskolin. See pp. 13726-13727, *Materials and Methods*. They teach that immunohistochemical analysis of embryoid bodies demonstrated that the cells could differentiate into a variety of cell types, including derivatives of the three embryonic germ layers. See p.

13729, 2^{nd} column, 1^{st} full ¶. They teach that these cellsl are karyotypically normal (see Abstract, and Material and Methods).

As Shamblott *et al.* teach human pluripotent stem cells which have a normal karyotype, they teach a disomic cell line, as required by the claims. Accordingly, Shamblott *et al.* anticipate the claimed invention.

Claims 1-2 are rejected under 35 U.S.C. 102(b) as being anticipated by Thomson *et al.* (**PNAS**, 92:7844-7848 (August 1995)).

The claims are directed to a disomic cell line derived from trisomic embryos, and a stem cell line derived from disomic cell lines.

Thomson *et al.* teach pluripotent primate embryonic stem cells, isolated from a rhesus monkey blastocyst. They teach that these cells remain undifferentiated in culture in continuous passage, maintain a normal karyotype, express appropriate cell markers [alkaline phosphatase, SSEA-3, SSEA-4, TRA-160-, TRA-1-81] and, when injected into SCID mice, they consistently differentiate into derivatives of all three germ layers. See *Abstract* and p. 7845-7846.

Accordingly, as Thomson teach a disomic stem cell line, they anticipate the claimed invention.

Claims 1-2 are rejected under 35 U.S.C. 102(b) as being anticipated by Thomson [**U.S. Pat. No.** 6,200,806 B1, March 13, 2001].

The claims are directed to a disomic cell line derived from trisomic embryos, and a stem cell line derived from disomic cell lines.

Thomson teach the preparation of a primate embryonic stem cell line that has expresses the cell surface markers characteristic of embryonic stem cells, have normal karyotypes, are able to proliferate in an undifferentiated state in continuous culture, and the ability to differentiate into all tissues derived from all three embryonic germ layers (see Abstract and claims).

Thus, because Thomson teach a karyotypically normal human embryonic stem cell line, they anticipate the claimed invention.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Thaian N. Ton whose telephone number is (571) 272-0736. The Examiner can normally be reached on Monday through Friday from 8:00 to 5:00 (Eastern Standard Time), with alternating Fridays off. Should the Examiner be unavailable, inquiries should be directed to Ram Shukla, SPE of Art Unit 1632, at (571) 272-0735. Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the Official Fax at (571) 273-8300. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989).

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

tnt
Thaian N. Ton
Patent Examiner
Group 1632

Anne-Marie Falk
-MARIE FALK, PH.D
RY EXAMINER

PTO/SB/08B (04-03)
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Application Number	10/625,100
Filing Date	
First Named Inventor	Santiago Munne
Art Unit	1632
Examiner Name	TON
Attorney Docket Number	

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Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
TNT	1	Munne S et al (2003) In: A color atlas of human assisted reproduction: clinical and ... pp179-194	
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Complete if Known	
Application Number	10/625,100
Filing Date	
First Named Inventor	Santiago Munne
Art Unit	1632
Examiner Name	TON
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TNT		US- 5843780	12-01-1998	Thompson	
TNT		US- 6335167	01-01-2002	Pinkel	
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Notice of References Cited	Application/Control No. 10/625,100	Applicant(s)/Patent Under Reexamination MUNNE, SANTIAGO	
	Examiner Thaian N. Ton	Art Unit 1632	Page 1 of 1

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*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,200,806 B1	03-2001	Thomson, James A.	435/366
	B	US-			
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	U	Shamblott et al. PNAS, 95:13726-13731 (1998).
	V	Thomson et al. PNAS, 92:7844-7848 (August 1995).
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(51) International Patent Classification 6 : C12N 5/00

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(30) Priority Data:
08/376,327 20 January 1995 (20.01.95) US

(71) Applicant: WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; 614 North Walnut Street, P.O. Box 7365, Madison, WI 53707-7365 (US).

(72) Inventor: THOMSON, James, A.; 2541 Fiedler Lane #3, Madison, WI 53713 (US).

(74) Agent: SEAY, Nicholas, J.; Quarles & Brady, P.O. Box 2113, Madison, WI 53701-2113 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

(54) Title: PRIMATE EMBRYONIC STEM CELLS

(57) Abstract

A purified preparation of primate embryonic stem cells is disclosed. This preparation is characterized by the following cell surface markers: SSEA-1 (-); SSEA-3 (+); SSEA-4 (+); TRA-1-60 (+); TRA-1-81 (+); and alkaline phosphatase (+). In a paricularly advantageous embodiment, the cells of the preparation have normal karyotypes and continue to proliferate in an undifferentiated state after continuous culture for eleven months. The embryonic stem cell lines also retain the ability, throughout the culture, to form trophoblast and to differentiate into all tissues derived from all three embryonic germ layers (endoderm, mesoderm and ectoderm). A method for isolating a primate embryonic stem cell line is also disclosed.

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PRIMATE EMBRYONIC STEM CELLS

Cross-Reference to Related Application

This application is a continuation-in-part of U.S. Application No. 08/376,327 filed January 20, 1995.

Field of the Invention

In general, the field of the present invention is stem cell cultures. Specifically, the field of the present invention is primate embryonic stem cell cultures.

Background of the Invention

In general, stem cells are undifferentiated cells which can give rise to a succession of mature functional cells. For example, a hematopoietic stem cell may give rise to any of the different types of terminally differentiated blood cells. Embryonic stem (ES) cells are derived from the embryo and are pluripotent, thus possessing the capability of developing into any organ or tissue type or, at least potentially, into a complete embryo.

One of the seminal achievements of mammalian embryology of the last decade is the routine insertion of specific genes into the mouse genome through the use of mouse ES cells. This alteration has created a bridge between the in vitro manipulations of molecular biology and an understanding of gene function in the intact animal. Mouse ES cells are undifferentiated,

pluripotent cells derived in vitro from preimplantation embryos (Evans, et al. Nature 292:154-159, 1981; Martin, Proc. Natl. Acad. Sci. USA 78:7634-7638, 1981) or from fetal germ cells (Matsui, et al., Cell 70:841-847, 1992). Mouse ES cells maintain an undifferentiated state through serial passages when cultured in the presence of fibroblast feeder layers in the presence of Leukemia Inhibitory Factor (LIF) (Williams, et al., Nature 336:684-687, 1988). If LIF is removed, mouse ES cells differentiate.

Mouse ES cells cultured in non-attaching conditions aggregate and differentiate into simple embryoid bodies, with an outer layer of endoderm and an inner core of primitive ectoderm. If these embryoid bodies are then allowed to attach onto a tissue culture surface, disorganized differentiation occurs of various cell types, including nerves, blood cells, muscle, and cartilage (Martin, 1981, supra; Doetschman, et al., J. Embryol. Exp. Morph. 87:27-45, 1985). Mouse ES cells injected into syngeneic mice form teratocarcinomas that exhibit disorganized differentiation, often with representatives of all three embryonic germ layers. Mouse ES cells combined into chimeras with normal preimplantation embryos and returned to the uterus participate in normal development (Richard, et al., Cytogenet. Cell Genet. 65:169-171, 1994).

The ability of mouse ES cells to contribute to functional germ cells in chimeras provides a method for introducing site-specific mutations into mouse lines. With appropriate transfection and selection strategies, homologous recombination can be used to derive ES cell lines with planned alterations of specific genes. These genetically altered cells can be used to form chimeras with normal embryos and chimeric animals are recovered. If the ES cells contribute to the germ line in the chimeric animal, then in the next

generation a mouse line for the planned mutation is established.

Because mouse ES cells have the potential to differentiate into any cell type in the body, mouse ES cells allow the in vitro study of the mechanisms controlling the differentiation of specific cells or tissues. Although the study of mouse ES cells provides clues to understanding the differentiation of general mammalian tissues, dramatic differences in primate and mouse development of specific lineages limits the usefulness of mouse ES cells as a model of human development. Mouse and primate embryos differ meaningfully in the timing of expression of the embryonic genome, in the formation of an egg cylinder versus an embryonic disc (Kaufman, The Atlas of Mouse Development, London: Academic Press, 1992), in the proposed derivation of some early lineages (O'Rahilly & Muller, Developmental Stages in Human Embryos, Washington: Carnegie Institution of Washington, 1987), and in the structure and function in the extraembryonic membranes and placenta (Mossman, Vertebrate Fetal Membranes, New Brunswick: Rutgers, 1987). Other tissues differ in growth factor requirements for development (e.g. the hematopoietic system(Lapidot et al., Lab An Sci 43:147-149, 1994)), and in adult structure and function (e.g. the central nervous system). Because humans are primates, and development is remarkably similar among primates, primate ES cells lines will provide a faithful model for understanding the differentiation of primate tissues in general and human tissues in particular.

The placenta provides just one example of how primate ES cells will provide an accurate model of human development that cannot be provided by ES cells from other species. The placenta and extraembryonic membranes differ dramatically between mice and humans. Structurally, the mouse placenta is classified as

labyrinthine, whereas the human and the rhesus monkey placenta are classified as villous. Chorionic gonadotropin, expressed by the trophoblast, is an essential molecule involved in maternal recognition of pregnancy in all primates, including humans (Hearn, J Reprod Fertil 76:809-819, 1986; Hearn et al., J Reprod Fert 92:497-509, 1991). Trophoblast secretion of chorionic gonadotropin in primates maintains the corpus luteum of pregnancy and, thus, progesterone secretion. Without progesterone, pregnancy fails. Yet mouse trophoblast produces no chorionic gonadotropin, and mice use entirely different mechanisms for pregnancy maintenance (Hearn et al., "Normal and abnormal embryo-fetal development in mammals," In: Lamming E, ed. Marshall's Physiology of Reproduction. 4th ed. Edinburgh, New York: Churchill Livingstone, 535-676, 1994). An immortal, euploid, primate ES cell line with the developmental potential to form trophoblast in vitro, will allow the study of the ontogeny and function of genes such as chorionic gonadotropin which are critically important in human pregnancy. Indeed, the differentiation of any tissue for which there are significant differences between mice and primates will be more accurately reflected in vitro by primate ES cells than by mouse ES cells.

The major in vitro models for studying trophoblast function include human choriocarcinoma cells, which are malignant cells that may not faithfully reflect normal trophectoderm; short-term primary cultures of human and non-human primate cytotrophoblast, which in present culture conditions quickly form non-dividing syncytial trophoblast; and in vitro culture of preimplantation non-human primate embryos (Hearn, et al., J. Endocrinol. 119:249-255, 1988; Coutifaris, et al., Ann. NY Acad. Sci. 191-201, 1994). An immortal, euploid, non-human primate embryonic stem (ES) cell line with the developmental

potential to form trophectoderm offers significant advantages over present in vitro models of human trophectoderm development and function, as trophoblast-specific genes such as chorionic gonadotropin could be stably altered in the ES cells and then studied during differentiation to trophectoderm.

The cell lines currently available that resembles primate ES cells most closely are human embryonic carcinoma (EC) cells, which are pluripotent, immortal cells derived from teratocarcinomas (Andrews, et al., Lab. Invest. 50(2):147-162, 1984; Andrews, et al., in: Robertson E., ed. Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. Oxford: IRL press, pp. 207-246, 1987). EC cells can be induced to differentiate in culture, and the differentiation is characterized by the loss of specific cell surface markers (SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81) and the appearance of new markers (Andrews, et al., 1987, supra). Human EC cells will form teratocarcinomas with derivatives of multiple embryonic lineages in tumors in nude mice. However, the range of differentiation of these human EC cells is limited compared to the range of differentiation obtained with mouse ES cells, and all EC cell lines derived to date are aneuploid (Andrews, et al., 1987, supra). Similar mouse EC cell lines have been derived from teratocarcinomas, and, in general their developmental potential is much more limited than mouse ES cells (Rossant, et al., Cell Differ. 15:155-161, 1984). Teratocarcinomas are tumors derived from germ cells, and although germ cells (like ES cells) are theoretically totipotent (i.e. capable of forming all cell types in the body), the more limited developmental potential and the abnormal karyotypes of EC cells are thought to result from selective pressures in the teratocarcinoma tumor environment

(Rossant & Papaioannou, Cell Differ 15:155-161, 1984). ES cells, on the other hand, are thought to retain greater developmental potential because they are derived from normal embryonic cells in vitro, without the selective pressures of the teratocarcinoma environment. Nonetheless, mouse EC cells and mouse ES cells share the same unique combination of cell surface markers (SSEA-1 (+), SSEA-3 (-), SSEA-4 (-), and alkaline phosphatase (+)).

Pluripotent cell lines have also been derived from preimplantation embryos of several domestic and laboratory animals species (Evans, et al., Theriogenology 33(1):125-128, 1990; Evans, et al., Theriogenology 33(1):125-128, 1990; Notarianni, et al., J. Reprod. Fertil. 41(Suppl.):51-56, 1990; Giles, et al., Mol. Reprod. Dev. 36:130-138, 1993; Graves, et al., Mol. Reprod. Dev. 36:424-433, 1993; Sukoyan, et al., Mol. Reprod. Dev. 33:418-431, 1992; Sukoyan, et al., Mol. Reprod. Dev. 36:148-158, 1993; Iannaccone, et al., Dev. Biol. 163:288-292, 1994).

Whether or not these cell lines are true ES cells lines is a subject about which there may be some difference of opinion. True ES cells should: (i) be capable of indefinite proliferation in vitro in an undifferentiated state; (ii) maintain a normal karyotype through prolonged culture; and (iii) maintain the potential to differentiate to derivatives of all three embryonic germ layers (endoderm, mesoderm, and ectoderm) even after prolonged culture. Strong evidence of these required properties have been published only for rodents ES cells including mouse (Evans & Kaufman, Nature 292:154-156, 1981; Martin, Proc Natl Acad Sci USA 78:7634-7638, 1981) hamster (Doetschmanet al. Dev Biol 127:224-227, 1988), and rat (Iannaccone et al. Dev Biol 163:288-292, 1994), and less conclusively for rabbit ES cells (Gileset al. Mol Reprod Dev 36:130-138, 1993; Graves & Moreadith, Mol

Reprod Dev 36:424-433, 1993). However, only established ES cell lines from the rat (Iannaccone, et al., 1994, supra) and the mouse (Bradley, et al., Nature 309:255-256, 1984) have been reported to participate in normal development in chimeras. There are no reports of the derivation of any primate ES cell line.

Summary of the Invention

The present invention is a purified preparation of primate embryonic stem cells. The primate ES cell lines are true ES cell lines in that they: (i) are capable of indefinite proliferation in vitro in an undifferentiated state; (ii) are capable of differentiation to derivatives of all three embryonic germ layers (endoderm, mesoderm, and ectoderm) even after prolonged culture; and (iii) maintain a normal karyotype throughout prolonged culture. The true primate ES cells lines are therefore pluripotent.

The present invention is also summarized in that primate ES cell lines are negative for the SSEA-1 marker, positive for the SSEA-3 marker, and positive for the SSEA-4 marker. Preferably, the primate ES cell lines are also positive for the TRA-1-60, and TRA-1-81 markers, as well as positive for the alkaline phosphatase marker.

It is an advantageous feature of the present invention that the primate ES cell lines continue to proliferate in an undifferentiated state after continuous culture for at least one year. In a particularly advantageous embodiment, the cells remain euploid after proliferation in an undifferentiated state.

It is a feature of the primate ES cell lines in accordance with the present invention that the cells can differentiate to trophoblast in vitro and express chorionic gonadotropin.

The present invention is also a purified preparation of primate embryonic stem cells that has the ability to differentiate into cells derived from mesoderm, endoderm, and ectoderm germ layers after the cells have been injected into an immunocompromised mouse, such as a SCID mouse.

The present invention is also a method of isolating a primate embryonic stem cell line. The method comprises the steps of isolating a primate blastocyst, isolating cells from the inner cellular mass (ICM) of the blastocyst, plating the ICM cells on a fibroblast layer (wherein ICM-derived cell masses are formed) removing an ICM-derived cell mass and dissociating the mass into dissociated cells, replating the dissociated cells on embryonic feeder cells and selecting colonies with compact morphology containing cells with a high nucleus/cytoplasm ratio, and prominent nucleoli. The cells of the selected colonies are then cultured.

It is an object of the present invention to provide a primate embryonic stem cell line.

It is an object of the present invention to provide a primate embryonic stem cell line characterized by the following markers: alkaline phosphatase(+); SSEA-1(-); SSEA-3(+); SSEA-4(+); TRA-1-60(+); and TRA-1-81(+).

It is an object of the present invention to provide a primate embryonic stem cell line capable of proliferation in an undifferentiated state after continuous culture for at least one year. Preferably, these cells remain euploid.

It is another object of the present invention to provide a primate embryonic stem cell line wherein the cells differentiate into cells derived from mesoderm, endoderm, and ectoderm germ layers when the cells are injected into an immunocompromised mouse.

Other objects, features, and advantages of the

present invention will become obvious after study of the specification, drawings, and claims.

Description of the Drawings

Fig. 1 is a photomicrograph illustrating normal XY karyotype of rhesus ES cell line R278.5 after 11 months of continuous culture.

Fig. 2 is a set of phase-contrast photomicrographs demonstrating the morphology of undifferentiated rhesus ES (R278.5) cells and of cells differentiated from R278.5 *in vitro* (bar = 100μ). Photograph A demonstrates the distinct cell borders, high nucleus to cytoplasm ratio, and prominent nucleoli of undifferentiated rhesus ES cells. Photographs B-D shows differentiated cells eight days after plating R278.5 cells on gel treated tissue culture plastic (with 10^3 units/ml added human LIF). Cells of these three distinct morphologies are consistently present when R278.5 cells are allowed to differentiate at low density without fibroblasts either in the presence or absence of soluble human LIF.

Fig. 3 are photomicrographs demonstrating the expression of cell surface markers on undifferentiated rhesus ES (R278.5) cells (bar = 100μ). Photograph A shows Alkaline Phosphatase (+); Photograph B shows SSEA-1 (-); Photograph C shows SSEA-3 (+); Photograph D shows SSEA-4 (+); Photograph E shows TRA-1-60 (+); and Photograph F shows TRA-1-81 (+).

Fig. 4 is a photograph illustrating expression of α-fetoprotein mRNA and α- and β- chorionic gonadotrophin mRNA expression in rhesus ES cells (R278.5) allowed to differentiate in culture.

Fig. 5 includes six photomicrographs of sections of tumors formed by injection of 0.5 X 10^6 rhesus ES (R278.5) cells into the hindleg muscles of SCID mice and analyzed 15 weeks later. Photograph A shows a low

power field demonstrating disorganized differentiation of multiple cell types. A gut-like structure is encircled by smooth muscle(s), and elsewhere foci of cartilage (c) are present (bar = 400μ); Photograph B shows striated muscle (bar = 40μ); Photograph C shows stratified squamous epithelium with several hair follicles. The labeled hair follicle (f) has a visible hair shaft (bar = 200μ); Photograph D shows stratified layers of neural cells in the pattern of a developing neural tube. An upper "ventricular" layer, containing numerous mitotic figures (arrows), overlies a lower "mantle" layer. (bar = 100μ); Photograph E shows ciliated columnar epithelium (bar = 40μ); Photograph F shows villi covered with columnar epithelium with interspersed mucus-secreting goblet cells (bar = 200μ).

Fig. 6 includes photographs of an embryoid Body. This embryoid body was formed from a marmoset ES cell line (Cj62) that had been continuously passaged <u>in vitro</u> for over 6 months. Photograph A (above) shows a section of the anterior 1/3 of the embryonic disc. Note the primitive ectoderm (E) forms a distinct cell layer from the underlying primitive endoderm (e), with no mixing of the cell layers. Note also that amnion (a) is composed of two distinct layers; the inner layer is continuous with the primitive ectoderm at the margins. Photograph B (below) shows a section in the caudal 1/3 of embryonic disc. Note central groove (arrow) and mixing of primitive ectoderm and endoderm representing early primitive streak formation, indicating the beginning of gastrulation. 400X, toluidine blue stain.

<u>Description of the Invention</u>

(1) <u>In General</u>

(a) <u>Uses of Primate ES Cells</u>

The present invention is a pluripotent, immortal

euploid primate ES cell line, as exemplified by the isolation of ES cell lines from two primate species, the common marmoset (*Callithrix jacchus*) and the rhesus monkey (*Macaca mulatta*). Primate embryonic stem cells are useful for:

(i) Generating transgenic non-human primates for models of specific human genetic diseases. Primate embryonic stem cells will allow the generation of primate tissue or animal models for any human genetic disease for which the responsible gene has been cloned. The human genome project will identify an increasing number of genes related to human disease, but will not always provide insights into gene function. Transgenic nonhuman primates will be essential for elucidating mechanisms of disease and for testing new therapies.

(ii) Tissue transplantation. By manipulating culture conditions, primate ES cells, human and non-human, can be induced to differentiate to specific cell types, such as blood cells, neuron cells, or muscle cells. Alternatively, primate ES cells can be allowed to differentiate in tumors in SCID mice, the tumors can be disassociated, and the specific differentiated cell types of interest can be selected by the usage of lineage specific markers through the use of fluorescent activated cell sorting (FACS) or other sorting method or by direct microdissection of tissues of interest. These differentiated cells could then be transplanted back to the adult animal to treat specific diseases, such as hematopoietic disorders, endocrine deficiencies, degenerative neurological disorders or hair loss.

(b) Selection of Model Species

Macaques and marmosets were used as exemplary species for isolation of a primate ES cell line. Macaques, such as the rhesus monkey, are Old World species that are the major primates used in biomedical

research. They are relatively large (about 7-10 kg). Males take 4-5 years to mature, and females have single young. Because of the extremely close anatomical and physiological similarities between humans and rhesus monkeys, rhesus monkey true ES cell lines provide a very accurate in vitro model for human differentiation. Rhesus monkey ES cell lines and rhesus monkeys will be particularly useful in the testing of the safety and efficacy of the transplantation of differentiated cell types into whole animals for the treatment of specific diseases or conditions. In addition, the techniques developed for the rhesus ES cell lines model the generation, characterization and manipulation of human ES cell lines.

The common marmoset (*Callithrix jacchus*) is a New World primate species with reproductive characteristics that make it an excellent choice for ES cell derivation. Marmosets are small (about 350-400 g), have a short gestation period (144 days), reach sexual maturity in about 18 months, and routinely have twins or triplets. Unlike in macaques, it is possible to routinely synchronize ovarian cycles in the marmoset with prostaglandin analogs, making collection of age-matched embryos from multiple females possible, and allowing efficient embryo transfer to synchronized recipients with 70%-80% of embryos transferred resulting in pregnancies. Because of these reproductive characteristics that allow for the routine efficient transfer of multiple embryos, marmosets provide an excellent primate species in which to generate transgenic models for human diseases.

There are approximately 200 primate species in the world. The most fundamental division that divides higher primates is between Old World and New world species. The evolutionary distance between the rhesus

monkey and the common marmoset is far greater than the evolutionary distance between humans and rhesus monkeys. Because it is here demonstrated that it is possible to isolate ES cell lines from a representative species of both the Old World and New World group using similar conditions, the techniques described below may be used successfully in deriving ES cell lines in other higher primates as well. Given the close evolutionary distance between rhesus macaques and humans, and the fact that feeder-dependent human EC cell lines can be grown in conditions similar to those that support primate ES cell lines, the same growth conditions will allow the isolation and growth of human ES cells. In addition, human ES cell lines will be permanent cell lines that will also be distinguished from all other permanent human cell lines by their normal karyotype and the expression of the same combination of cell surface markers (alkaline phosphotase, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81) that characterize other primate ES cell lines. A normal karyotype and the expression of this combination of cell surface markers will be defining properties of true human ES cell lines, regardless of the method used for their isolation and regardless of their tissue of origin.

No other primate (human or non-human) ES cell line is known to exist. The only published permanent, euploid, embryo-derived cell lines that have been convincingly demonstrated to differentiate into derivatives of all three germ layers have been derived from rodents (the mouse, rat, and hamster), and possibly from rabbit. The published reports of embryo-derived cell lines from domestic species have failed to convincingly demonstrate differentiation of derivatives of all three embryonic germ layers or have not been permanent cell lines. Research groups in Britain and Singapore are informally reported, later

than the work described here, to have attempted to derive human ES cell lines from surplus in vitro fertilization-produced human embryos, although they have not yet reported success in demonstrating pluripotency of their cells and have failed to isolate permanent cell lines. In the only published report on attempts to isolate human ES cells, conditions were used (LIF in the absence of fibroblast feeder layers) that the results below will indicate will not result in primate ES cells which can remain in an undifferentiated state. It is not surprising, then that the cells grown out of human ICMs failed to continue to proliferate after 1 or 2 subcultures, Bongso et al. Hum. Reprod. 9:2100-2117 (1994).

(2) Embryonic Stem Cell Isolation

A preferable medium for isolation of embryonic stem cells is "ES medium." ES medium consists of 80% Dulbecco's modified Eagle's medium (DMEM; no pyruvate, high glucose formulation, Gibco BRL), with 20% fetal bovine serum (FBS; Hyclone), 0.1 mM β-mercaptoethanol (Sigma), 1% non-essential amino acid stock (Gibco BRL). Preferably, fetal bovine serum batches are compared by testing clonal plating efficiency of a low passage mouse ES cell line (ES_{jt3}), a cell line developed just for the purpose of this test. FBS batches must be compared because it has been found that batches vary dramatically in their ability to support embryonic cell growth, but any other method of assaying the competence of FBS batches for support of embryonic cells will work as an alternative.

Primate ES cells are isolated on a confluent layer of murine embryonic fibroblast in the presence of ES cell medium. Embryonic fibroblasts are preferably obtained from 12 day old fetuses from outbred CF1 mice (SASCO), but other strains may be used as an alternative. Tissue culture dishes are

preferably treated with 0.1% gelatin (type I; Sigma).

For rhesus monkey embryos, adult female rhesus monkeys (greater than four years old) demonstrating normal ovarian cycles are observed daily for evidence of menstrual bleeding (day 1 of cycle = the day of onset of menses). Blood samples are drawn daily during the follicular phase starting from day 8 of the menstrual cycle, and serum concentrations of luteinizing hormone are determined by radioimmunoassay. The female is paired with a male rhesus monkey of proven fertility from day 9 of the menstrual cycle until 48 hours after the luteinizing hormone surge; ovulation is taken as the day following the luteinizing hormone surge. Expanded blastocysts are collected by non-surgical uterine flushing at six days after ovulation. This procedure routinely results in the recovery of an average 0.4 to 0.6 viable embryos per rhesus monkey per month, Seshagiri et al. Am J Primatol 29:81-91, 1993.

For marmoset embryos, adult female marmosets (greater than two years of age) demonstrating regular ovarian cycles are maintained in family groups, with a fertile male and up to five progeny. Ovarian cycles are controlled by intramuscular injection of 0.75 g of the prostaglandin PGF2a analog cloprostenol (Estrumate, Mobay Corp, Shawnee, KS) during the middle to late luteal phase. Blood samples are drawn on day 0 (immediately before cloprostenol injection), and on days 3, 7, 9, 11, and 13. Plasma progesterone concentrations are determined by ELISA. The day of ovulation is taken as the day preceding a plasma progesterone concentration of 10 ng/ml or more. At eight days after ovulation, expanded blastocysts are recovered by a non-surgical uterine flush procedure, Thomson et al. "Non-surgical uterine stage preimplantation embryo collection from the common marmoset," J Med Primatol, 23:333-336 (1994). This

procedure results in the average production of 1.0 viable embryos per marmoset per month.

The zona pellucida is removed from blastocysts by brief exposure to pronase (Sigma). For immunosurgery, blastocysts are exposed to a 1:50 dilution of rabbit anti-marmoset spleen cell antiserum (for marmoset blastocysts) or a 1:50 dilution of rabbit anti-rhesus monkey (for rhesus monkey blastocysts) in DMEM for 30 minutes, then washed for 5 minutes three times in DMEM, then exposed to a 1:5 dilution of Guinea pig complement (Gibco) for 3 minutes.

After two further washes in DMEM, lysed trophectoderm cells are removed from the intact inner cell mass (ICM) by gentle pipetting, and the ICM plated on mouse inactivated (3000 rads gamma irradiation) embryonic fibroblasts.

After 7-21 days, ICM-derived masses are removed from endoderm outgrowths with a micropipette with direct observation under a stereo microscope, exposed to 0.05% Trypsin-EDTA (Gibco) supplemented with 1% chicken serum for 3-5 minutes and gently dissociated by gentle pipetting through a flame polished micropipette.

Dissociated cells are replated on embryonic feeder layers in fresh ES medium, and observed for colony formation. Colonies demonstrating ES-like morphology are individually selected, and split again as described above. The ES-like morphology is defined as compact colonies having a high nucleus to cytoplasm ratio and prominent nucleoli. Resulting ES cells are then routinely split by brief trypsinization or exposure to Dulbecco's Phosphate Buffered Saline (without calcium or magnesium and with 2 mM EDTA) every 1-2 weeks as the cultures become dense. Early passage cells are also frozen and stored in liquid nitrogen.

Cell lines may be karyotyped with a standard G-

banding technique (such as by the Cytogenetics Laboratory of the University of Wisconsin State Hygiene Laboratory, which provides routine karyotyping services) and compared to published karyotypes for the primate species.

Isolation of ES cell lines from other primate species would follow a similar procedure, except that the rate of development to blastocyst can vary by a few days between species, and the rate of development of the cultured ICMs will vary between species. For example, six days after ovulation, rhesus monkey embryos are at the expanded blastocyst stage, whereas marmoset embryos don't reach the same stage until 7-8 days after ovulation. The Rhesus ES cell lines were obtained by splitting the ICM-derived cells for the first time at 7-16 days after immunosurgery; whereas the marmoset ES cells were derived with the initial split at 7-10 days after immunosurgery. Because other primates also vary in their developmental rate, the timing of embryo collection, and the timing of the initial ICM split will vary between primate species, but the same techniques and culture conditions will allow ES cell isolation.

Because ethical considerations in the U.S. do not allow the recovery of human <u>in vivo</u> fertilized preimplantation embryos from the uterus, human ES cells that are derived from preimplantation embryos will be derived from <u>in vitro</u> fertilized (IVF) embryos. Experiments on unused (spare) human IVF-produced embryos are allowed in many countries, such as Singapore and the United Kingdom, if the embryos are less than 14 days old. Only high quality embryos are suitable for ES isolation. Present defined culture conditions for culturing the one cell human embryo to the expanded blastocyst are suboptimal but practicable, Bongso et al., <u>Hum Reprod</u> 4:706-713, 1989. Co-culturing of human embryos with human

oviductal cells results in the production of high blastocyst quality. IVF-derived expanded human blastocysts grown in cellular co-culture, or in improved defined medium, will allow the isolation of human ES cells with the same procedures described above for nonhuman primates.

(3) Defining Characteristics of Primate ES Cells

Primate embryonic stem cells share features with the primate ICM and with pluripotent human embryonal carcinoma cells. Putative primate ES cells may therefore be characterized by morphology and by the expression of cell surface markers characteristic of human EC cells. Additionally, putative primate ES cells may be characterized by developmental potential, karyotype and immortality.

(a) Morphology

The colony morphology of primate embryonic stem cell lines is similar to, but distinct from, mouse embryonic stem cells. Both mouse and primate ES cells have the characteristic features of undifferentiated stem cells, with high nuclear/cytoplasmic ratios, prominent nucleoli, and compact colony formation. The colonies of primate ES cells are flatter than mouse ES cell colonies and individual primate ES cells can be easily distinguished. In Fig. 2, reference character A indicates a phase contrast photomicrograph of cell line R278.5 demonstrating the characteristic primate ES cell morphology.

(b) Cell Surface Markers

A primate ES cell line of the present invention is distinct from mouse ES cell lines by the presence or absence of the cell surface markers described below.

One set of glycolipid cell surface markers is known as the Stage-specific embryonic antigens 1 through 4. These antigens can be identified using

antibodies for SSEA 1, SSEA-3 and SSEA-4 which are available from the Developmental Studies Hybridoma Bank of the National Institute of Child Health and Human Development. The cell surface markers referred to as TRA-1-60 and TRA-1-81 designate antibodies from hybridomas developed by Peter Andrews of the University of Sheffield and are described in Andrews et al., "Cell lines from human germ cell tumors," In: Robertson E, ed. Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. Oxford: IRL Press, 207-246, 1987. The antibodies were localized with a biotinylated secondary antibody and then an avidin/biotinylated horseradish peroxidase complex (Vectastain ABC System, Vector Laboratories). Alternatively, it should also be understood that other antibodies for these same cell surface markers can be generated. NTERA-2 cl. D1, a pluripotent human EC cell line (gift of Peter Andrews), may be used as a negative control for SSEA-1, and as a positive control for SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. This cell line was chosen for positive control only because it has been extensively studied and reported in the literature, but other human EC cell lines may be used as well.

Mouse ES cells (ES_{jt3}) are used as a positive control for SSEA-1, and for a negative control for SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81. Other routine negative controls include omission of the primary or secondary antibody and substitution of a primary antibody with an unrelated specificity.

Alkaline phosphatase may be detected following fixation of cells with 4% para-formaldehyde using "Vector Red" (Vector Laboratories) as a substrate, as described by the manufacturer (Vector Laboratories). The precipitate formed by this substrate is red when viewed with a rhodamine filter system, providing substantial amplification over light microscopy.

Table 1 diagrams a comparison of mouse ES cells, primate ES cells, and human EC cells. The only cells reported to express the combination of markers SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 other than primate ES cells are human EC cells. The globo-series glycolipids SSEA-3 and SSEA-4 are consistently present on human EC cells, and are of diagnostic value in distinguishing human EC cell tumors from human yolk sac carcinomas, choriocarcinomas, and other lineages which lack these markers, Wenk et al., Int J Cancer 58:108-115, 1994. A recent survey found SSEA-3 and SSEA-4 to be present on all of over 40 human EC cell lines examined, Wenk et al. TRA-1-60 and TRA-1-81 antigens have been studied extensively on a particular pluripotent human EC cell line, NTERA-2 CL. D1, Andrews et al, supra. Differentiation of NTERA-2 CL. D1 cells in vitro results in the loss of SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 expression and the increased expression of the lacto-series glycolipid SSEA-1, Andrews et al, supra. This contrasts with undifferentiated mouse ES cells, which express SSEA-1, and neither SSEA-3 nor SSEA-4. Although the function of these antigens are unknown, their shared expression by R278.5 cells and human EC cells suggests a close embryological similarity. Alkaline phosphatase will also be present on all primate ES cells. A successful primate ES cell culture of the present invention will correlate with the cell surface markers found in the rhesus macaque and marmoset cell lines described in Table 1.

As disclosed below in Table 1, the rhesus macaque and marmoset cell lines are identical to human EC cell lines for the 5 described markers. Therefore, a successful primate ES cell culture will also mimic human EC cells. However, there are other ways to discriminate ES cells from EC cells. For example, the primate ES cell line has a normal karyotype and the

human EC cell line is aneuploid.

In Fig. 3, the photographs labelled A through F demonstrate the characteristic staining of these markers on a rhesus monkey ES cell line designated R278.5.

Table 1

	Mouse ES	C. jacchus ES	M. mulatta ES	Human EC (NTERA-2 cl.D1)
SSEA-1	+	-	-	-
SSEA-3	-	+	+	+
SSEA-4	-	+	+	+
Tra-1-60	-	+	+	+
Tra-1-81	-	+	+	+

(c) Developmental Potential

Primate ES cells of the present invention are pluripotent. By "pluripotent" we mean that the cell has the ability to develop into any cell derived from the three main germ cell layers or an embryo itself. When injected into SCID mice, a successful primate ES cell line will differentiate into cells derived from all three embryonic germ layers including: bone, cartilage, smooth muscle, striated muscle, and hematopoietic cells (mesoderm); liver, primitive gut and respiratory epithelium (endoderm); neurons, glial cells, hair follicles, and tooth buds (ectoderm).

This experiment can be accomplished by injecting approximately 0.5-1.0 X 10^6 primate ES cells into the rear leg muscles of 8-12 week old male SCID mice. The resulting tumors can be fixed in 4% paraformaldehyde and examined histologically after paraffin embedding at 8-16 weeks of development. In Fig. 4, photomicrographs designated A-F are of sections of tumors formed by injection of rhesus ES cells into the hind leg muscles of SCID mice and analyzed 15 weeks later demonstrating cartilage, smooth muscle, and striated muscle (mesoderm); stratified squamous epithelium with hair follicles,

neural tube with ventricular, intermediate, and mantle layers (ectoderm) ; ciliated columnar epithelium and villi lined by absorptive enterocytes and mucus-secreting goblet cells (endoderm).

A successful nonhuman primate ES cell line will have the ability to participate in normal development when combined in chimeras with normal preimplantation embryos. Chimeras between preimplantation nonhuman primate embryos and nonhuman primate ES cells can be formed by routine methods in several ways. (i) injection chimeras: 10-15 nonhuman primate ES cells can be microinjected into the cavity of an expanded nonhuman primate blastocyst; (ii) aggregation chimeras: nonhuman primate morulae can be co-cultured on a lawn of nonhuman primate ES cells and allowed to aggregate; and (iii) tetraploid chimeras: 10-15 nonhuman primate ES cells can be aggregated with tetraploid nonhuman primate morulae obtained by electrofusion of 2-cell embryos, or incubation of morulae in the cytoskeletal inhibitor cholchicine. The chimeras can be returned to the uterus of a female nonhuman primate and allowed to develop to term, and the ES cells will contribute to normal differentiated tissues derived from all three embryonic germ layers and to germ cells. Because nonhuman primate ES can be genetically manipulated prior to chimera formation by standard techniques, chimera formation followed by embryo transfer can lead to the production of transgenic nonhuman primates.

(d) <u>Karyotype</u>

Successful primate ES cell lines have normal karyotypes. Both XX and XY cells lines will be derived. The normal karyotypes in primate ES cell lines will be in contrast to the abnormal karyotype found in human embryonal carcinoma (EC), which are derived from spontaneously arising human germ cell

tumors (teratocarcinomas). Human embryonal carcinoma cells have a limited ability to differentiate into multiple cell types and represent the closest existing cell lines to primate ES cells. Although tumor-derived human embryonal carcinoma cell lines have some properties in common with embryonic stem cell lines, all human embryonal carcinoma cell lines derived to date are aneuploid. Thus, primate ES cell lines and human EC cell lines can be distinguished by the normal karyotypes found in primate ES cell lines and the abnormal karyotypes found in human EC lines. By "normal karyotype" it is meant that all chromosomes normally characteristic of the species are present and have not been noticeably altered.

Because of the abnormal karyotypes of human embryonal carcinoma cells, it is not clear how accurately their differentiation reflects normal differentiation. The range of embryonic and extra-embryonic differentiation observed with primate ES cells will typically exceed that observed in any human embryonal carcinoma cell line, and the normal karyotypes of the primate ES cells suggests that this differentiation accurately recapitulates normal differentiation.

(e) Immortality

Immortal cells are capable of continuous indefinite replication in vitro. Continued proliferation for longer than one year of culture is a sufficient evidence for immortality, as primary cell cultures without this property fail to continuously divide for this length of time (Freshney, Culture of animal cells. New York: Wiley-Liss, 1994). Primate ES cells will continue to proliferate in vitro with the culture conditions described above for longer than one year, and will maintain the developmental potential to contribute all three embryonic germ layers. This developmental

potential can be demonstrated by the injection of ES cells that have been cultured for a prolonged period (over a year) into SCID mice and then histologically examining the resulting tumors. Although karyotypic changes can occur randomly with prolonged culture, some primate ES cells will maintain a normal karyotype for longer than a year of continuous culture.

(f) Culture Conditions

Growth factor requirements to prevent differentiation are different for the primate ES cell line of the present invention than the requirements for mouse ES cell lines. In the absence of fibroblast feeder layers, Leukemia inhibitory factor (LIF) is necessary and sufficient to prevent differentiation of mouse ES cells and to allow their continuous passage. Large concentrations of cloned LIF fail to prevent differentiation of primate ES cell lines in the absence of fibroblast feeder layers. In this regard, primate ES stem cells are again more similar to human EC cells than to mouse ES cells, as the growth of feeder-dependent human EC cells lines is not supported by LIF in the absence of fibroblasts.

(g) Differentiation to Extra Embryonic Tissues

When grown on embryonic fibroblasts and allowed to grow for two weeks after achieving confluence (i.e., continuously covering the culture surface), primate ES cells of the present invention spontaneously differentiate and will produce chorionic gonadotropin, indicating trophoblast differentiation (a component of the placenta) and produce α-fetoprotein, indicating endoderm differentiation. Chorionic gonadotropin activity can be assayed in the medium conditioned by differentiated cells by Leydig cell bioassay, Seshagiri & Hearn, Hum Reprod 8:279-287, 1992. For

mRNA analysis, RNA can be prepared by guanidine isothiocyanate-phenol/chloroform extraction (1) from approximately 0.2 X 10^6 differentiated cells and from 0.2 X 10^6 undifferentiated cells. The relative levels of the mRNA for α-fetoprotein and the α- and β-subunit of chorionic gonadotropin relative to glyceraldehyde-3-phosphate dehydrogenase can be determined by semi-quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The PCR primers for glyceraldehyde 3-phosphate dehydrogenase (G3PDH), obtained from Clontech (Palo Alto, CA), are based on the human cDNA sequence, and do not amplify mouse G3PDH mRNA under our conditions. Primers for the α-fetoprotein mRNA are based on the human sequence and flank the 7th intron (5' primer = (5') GCTGGATTGTCTGCAGGATGGGGAA (SEQ ID NO: 1); 3' primer = (5') TCCCCTGAAGAAAATTGGTTAAAAT (SEQ ID NO: 2)). They amplify a cDNA of 216 nucleotides. Primers for the β-subunit of chorionic gonadotropin flank the second intron (5' primer = (5') ggatc CACCGTCAACACCACCATCTGTGC (SEQ ID NO: 3); 3' primer = (5') ggatc CACAGGTCAAAGGGTGGTCCTTGGG (SEQ ID NO: 4)) (nucleotides added to the hCGb sequence to facilitate sub-cloning are shown in lower case italics). They amplify a cDNA of 262 base pairs. The primers for the CGα subunit can be based on sequences of the first and fourth exon of the rhesus gene (5' primer = (5') gggaattc GCAGTTACTGAGAACTCACAAG (SEQ ID NO: 5); 3' primer = (5') gggaattc GAAGCATGTCAAAGTGGTATGG (SEQ ID NO: 6)) and amplify a cDNA of 556 base pairs. The identity of the α-fetoprotein, CGα and CGβ cDNAs can be verified by subcloning and sequencing.

For Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), 1 to 5 μl of total R278.5 RNA can be reverse transcribed as described Golos et al. <u>Endocrinology</u> 133(4):1744-1752, 1993, and one to 20 μl of reverse transcription reaction was then

subjected to the polymerase chain reaction in a mixture containing 1-12.5 pmol of each G3PDH primer, 10-25 pmol of each mRNA specific primer, 0.25 mM dNTPs (Pharmacia, Piscataway, NJ), 1X AmpliTaq buffer (final reaction concentrations = 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% (w/v) gelatin) 2.5 μCi of deoxycytidine 5'a[32P]triphosphate (DuPont, Boston, MA), 10% glycerol and 1.25 U of AmpliTaq (Perkin-Elmer, Oak Brook, IL) in a total volume of 50 μl. The number of amplification rounds which produced linear increases in target cDNAs and the relation between input RNA and amount of PCR product is empirically determined as by Golos et al. Samples were fractionated in 3% Nusieve (FMC, Rockland, ME) agarose gels (1X TBE running buffer) and DNA bands of interest were cut out, melted at 65°C in 0.5 ml TE, and radioactivity determined by liquid scintillation counting. The ratio of counts per minute in a specific PCR product relative to cpm of G3PDH PCR product is used to estimate the relative levels of a mRNAs among differentiated and undifferentiated cells.

The ability to differentiate into trophectoderm in vitro and the ability of these differentiated cells to produce chorionic gonadotropin distinguishes the primate ES cell line of the present invention from all other published ES cell lines.

Examples

(1) Animals and Embryos

As described above, we have developed a technique for non-surgical, uterine-stage embryo recovery from the rhesus macaque and the common marmoset.

To supply rhesus embryos to interested investigators, The Wisconsin Regional Primate Research Center (WRPRC) provides a preimplantation

embryo recovery service for the rhesus monkey, using the non-surgical flush procedure described above. During 1994, 151 uterine flushes were attempted from rhesus monkeys, yielding 80 viable embryos (0.53 embryos per flush attempt).

By synchronizing the reproductive cycles of several marmosets, significant numbers of *in vivo* produced, age-matched, preimplantation primate embryos were studied in controlled experiments for the first time. Using marmosets from the self-sustaining colony (250 animals) of the Wisconsin Regional Primate Research Center (WRPRC), we recovered 54 viable morulae or blastocysts, 7 unfertilized oocytes or degenerate embryos, and 5 empty zonae pellucidae in a total of 54 flush attempts (1.0 viable embryo-flush attempt). Marmosets have a 28 day ovarian cycle, and because this is a non-surgical procedure, females can be flushed on consecutive months, dramatically increasing the embryo yield compared to surgical techniques which require months of rest between collections.

(2) <u>Rhesus Macaque Embryonic Stem Cells</u>

Using the techniques described above, we have derived three independent embryonic stem cell lines from two rhesus monkey blastocysts (R278.5, R366, and R367). One of these, R278.5, remains undifferentiated and continues to proliferate after continuous culture for over one year. R278.5 cells have also been frozen and successfully thawed with the recovery of viable cells.

The morphology and cell surface markers of R278.5 cells are indistinguishable from human EC cells, and differ significantly from mouse ES cells. R278.5 cells have a high nucleus/cytoplasm ratio and prominent nucleoli, but rather than forming compact, piled-up colonies with indistinct cell borders similar to mouse ES cells, R278.5 cells form flatter

colonies with individual, distinct cells (Fig 2 A). R278.5 cells express the SSEA-3, SSEA-4, TRA-1-60, and TRA-81 antigens (Fig 3 and Table 1), none of which are expressed by mouse ES cells. The only cells known to express the combination of markers SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 other than primate ES cells are human EC cells. The globo-series glycolipids SSEA-3 and SSEA-4 are consistently present on human EC cells, and are of diagnostic value in distinguishing human EC cell tumors from yolk sac carcinomas, choriocarcinomas and other stem cells derived from human germ cell tumors which lack these markers, Wenk et al, Int J Cancer 58:108-115, 1994. A recent survey found SSEA-3 and SSEA-4 to be present on all of over 40 human EC cell lines examined (Wenk et al.).

TRA-1-60 and TRA-1-81 antigens have been studied extensively on a particular pluripotent human EC cell line, NTERA-2 CL. D1 (Andrews et al.). Differentiation of NTERA-2 CL. D1 cells in vitro results in the loss of SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 expression and the increased expression of the lacto-series glycolipid SSEA-1. Undifferentiated mouse ES cells, on the other hand, express SSEA-1, and not SSEA-3, SSEA-4, TRA-1-60 or TRA-1-81 (Wenk et al.). Although the function of these antigens is unknown, their expression by R278.5 cells suggests a close embryological similarity between primate ES cells and human EC cells, and fundamental differences between primate ES cells and mouse ES cells.

R278.5 cells also express alkaline phosphatase. The expression of alkaline phosphatase is shared by both primate and mouse ES cells, and relatively few embryonic cells express this enzyme. Positive cells include the ICM and primitive ectoderm (which are the most similar embryonic cells in the intact embryo to ES cells), germ cells, (which are totipotent), and a

very limited number of neural precursors, Kaufman MH. The atlas of mouse development. London: Academic Press, 1992. Cells not expressing this enzyme will not be primate ES cells.

Although cloned human LIF was present in the medium at cell line derivation and for initial passages, R278.5 cells grown on mouse embryonic fibroblasts without exogenous LIF remain undifferentiated and continued to proliferate. R278.5 cells plated on gelatin-treated tissue culture plates without fibroblasts differentiated to multiple cell types or failed to attach and died, regardless of the presence or absence of exogenously added human LIF (Fig 2). Up to 10^4 units/ ml human LIF fails to prevent differentiation. In addition, added LIF fails to increase the cloning efficiency or proliferation rate of R278.5 cells on fibroblasts. Since the derivation of the R278.5 cell line, we have derived two additional rhesus ES cell lines (R366 and R367) on embryonic fibroblasts without any exogenously added LIF at initial derivation. R366 and R367 cells, like R278.5 cells, continue to proliferate on embryonic fibroblasts without exogenously added LIF and differentiate in the absence of fibroblasts, regardless of the presence of added LIF. RT-PCR performed on mRNA from spontaneously differentiated R278.5 cells revealed α-fetoprotein mRNA (Fig 4). α-fetoprotein is a specific marker for endoderm, and is expressed by both extra-embryonic (yolk sac) and embryonic (fetal liver and intestines) endoderm-derived tissues. Epithelial cells resembling extraembryonic endoderm are present in cells differentiated in vitro from R278.5 cells (Fig. 2). Bioactive CG (3.89 mI units/ml) was present in culture medium collected from differentiated cells, but not in medium collected from undifferentiated cells (less than 0.03

mI units/ml), indicating the differentiation of trophoblast, a trophectoderm derivative. The relative level of the CGα mRNA increased 23.9-fold after differentiation (Fig. 4).

All SCID mice injected with R278.5 cells in either intra-muscular or intra-testicular sites formed tumors, and tumors in both sites demonstrated a similar range of differentiation. The oldest tumors examined (15 weeks) had the most advanced differentiation, and all had abundant, unambiguous derivatives of all three embryonic germ layers, including gut and respiratory epithelium (endoderm); bone, cartilage, smooth muscle, striated muscle (mesoderm); ganglia, glia, neural precursors, and stratified squamous epithelium (ectoderm), and other unidentified cell types (Fig. 5). In addition to individual cell types, there was organized development of some structures which require complex interactions between different cell types. Such structures included gut lined by villi with both absorptive enterocytes and mucus-secreting goblet cells, and sometimes encircled by layers of smooth muscle in the same orientation as muscularis mucosae (circular) and muscularis (outer longitudinal layer and inner circular layer); neural tubes with ventricular, intermediate, and mantle layers; and hair follicles with hair shafts (Fig. 5).

The essential characteristics that define R278.5 cells as ES cells include: indefinite (greater than one year) undifferentiated proliferation <u>in vitro</u>, normal karyotype, and potential to differentiate to derivatives of trophectoderm and all three embryonic germ layers. In the mouse embryo, the last cells capable of contributing to derivatives of both trophectoderm and ICM are early ICM cells. The timing of commitment to ICM or trophectoderm has not been established for any primate species, but the

potential of rhesus ES cells to contribute to derivatives of both suggests that they most closely resemble early totipotent embryonic cells. The ability of rhesus ES cells to form trophoblast in vitro distinguishes primate ES cell lines from mouse ES cells. Mouse ES cell have not been demonstrated to form trophoblast in vitro, and mouse trophoblast does not produce gonadotropin. Rhesus ES cells and mouse ES cells do demonstrate the similar wide range of differentiation in tumors that distinguishes ES cells from EC cells. The development of structures composed of multiple cell types such as hair follicles, which require inductive interactions between the embryonic epidermis and underlying mesenchyme, demonstrates the ability of rhesus ES cells to participate in complex developmental processes.

The rhesus ES lines R366 and R367 have also been further cultured and analyzed. Both lines have a normal XY karyotype and were proliferated in an undifferentiated state for about three months prior to freezing for later analysis. Samples of each of the cell lines R366 and R367 were injected into SCID mice which then formed teratomas identical to those formed by R278.5 cells. An additional rhesus cell line R394 having a normal XX karyotype was also recovered. All three of these cell lines, R366, R367 and R394 are identical in morphology, growth characteristics, culture requirements and in vitro differentiation characteristics, i.e. the trait of differentiation to multiple cell types in the absence of fibroblasts, to cell line 278.5.

It has been determined that LIF is not required either to derive or proliferate these ES cultures. Each of the cell lines R366, R367 and R394 were derived and cultured without exogenous LIF.

It has also been demonstrated that the

particular source of fibroblasts for co-culture is not critical. Several fibroblast cell lines have been tested both with rhesus line R278.5 and with the marmoset cell lines described below. The fibroblasts tested include mouse STO cells (ATCC 56-X), mouse 3T3 cells (ATCC 48-X), primary rhesus monkey embryonic fibroblasts derived from 36 day rhesus fetuses, and mouse Sl/Sl^4 cells, which are deficient in the steel factor. All these fibroblast cell lines were capable of maintaining the stem cell lines in an undifferentiated state. Most rapid proliferation of the stem cells was observed using primary mouse embryonic fibroblasts.

Unlike mouse ES cells, neither rhesus ES cells nor feeder-dependent human EC cells remain undifferentiated and proliferate in the presence of soluble human LIF without fibroblasts. The factors that fibroblasts produce that prevent the differentiation of rhesus ES cells or feeder-dependent human EC cells are unknown, but the lack of a dependence on LIF is another characteristic that distinguishes primate ES cells from mouse ES cells. The growth of rhesus monkey ES cells in culture conditions similar to those required by feeder-dependent human EC cells, and the identical morphology and cell surface markers of rhesus ES cells and human EC cells, suggests that similar culture conditions will support human ES cells.

Rhesus ES cells will be important for elucidating the mechanisms that control the differentiation of specific primate cell types. Given the close evolutionary distance and the developmental and physiological similarities between humans and rhesus monkeys, the mechanisms controlling the differentiation of rhesus cells will be very similar to the mechanisms controlling the differentiation of human cells. The importance of

elucidating these mechanisms is that once they are understood, it will be possible to direct primate ES cells to differentiate to specific cell types *in vitro*, and these specific cell types can be used for transplantation to treat specific diseases.

Because ES cells have the developmental potential to give rise to any differentiated cell type, any disease that results in part or in whole from the failure (either genetic or acquired) of specific cell types will be potentially treatable through the transplantation of cells derived from ES cells. Rhesus ES cells and rhesus monkeys will be invaluable for testing the efficacy and safety of the transplantation of specific cell types derived from ES cells. A few examples of human diseases potentially treatable by this approach with human ES cells include degenerative neurological disorders such as Parkinson's disease (dopanergic neurons), juvenile onset diabetes (pancreatic β-islet cells) or Acquired Immunodeficiency Disease (lymphocytes). Because undifferentiated ES cells can proliferate indefinitely *in vitro*, they can be genetically manipulated with standard techniques either to prevent immune rejection after transplantation, or to give them new genetic properties to combat specific diseases. For specific cell types where immune rejection can be prevented, cells derived from rhesus monkey ES cells or other non-human primate ES cells could be used for transplantation to humans to treat specific diseases.

(3) Marmoset Embryonic Stem Cells

Our method for creating an embryonic stem cell line is described above. Using isolated ICM's derived by immunosurgery from marmoset blastocysts, we have isolated 7 putative ES cell lines, each of which have been cultured for over 6 months.

One of these, Cj11, was cultured continuously for over 14 months, and then frozen for later analysis. The Cj11 cell line and other marmoset ES cell lines have been successfully frozen and then thawed with the recovery of viable cells. These cells have a high nuclear/cytoplasmic ratio, prominent nucleoli, and a compact colony morphology similar to the pluripotent human embryonal carcinoma (EC) cell line NT2/D2.

Four of the cell lines we have isolated have normal XX karyotypes, and one has a normal XY karyotype (Karyotypes were performed by Dr. Charles Harris, University of Wisconsin). These cells were positive for a series of cell surface markers (alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81) that in combination are definitive markers for undifferentiated human embryonal carcinoma cells (EC) cells and primate ES cells. In particular, these markers distinguish EC cells from the earliest lineages to differentiate in the human preimplantation embryo, trophectoderm (represented by BeWO choriocarcinoma cells) and extraembryonic endoderm (represented by 1411H yolk sac carcinoma cells).

When the putative marmoset ES cells were removed from fibroblast feeders, they differentiated into cells of several distinct morphologies. Among the differentiated cells, trophectoderm is indicated by the secretion of chorionic gonadotropin and the presence of the chorionic gonadotropin β-subunit mRNA. 12.7 mIU/ml luteinizing hormone (LH) activity was measured in the WRPRC core assay lab using a mouse Leydig cell bioassay in medium conditioned 24 hours by putative ES cells allowed to differentiate for one week. Note that chorionic gonadotrophin has both LH and FSH activity, and is routinely measured by LH assays. Control medium from undifferentiated

ES cells had less than 1 mIU/ml LH activity.

Chorionic gonadotropin β-subunit mRNA was detected by reverse transcriptase-polymerase chain reaction (RT-PCR). DNA sequencing confirmed the identity of the chorionic gonadotrophin β-subunit.

Endoderm differentiation (probably extraembryonic endoderm) was indicated by the presence of α-fetoprotein mRNA, detected by RT-PCR.

When the marmoset ES cells were grown in high densities, over a period of weeks epithelial cells differentiated and covered the culture dish. The remaining groups of undifferentiated cells rounded up into compact balls and then formed embryoid bodies (as shown in Fig. 6) that recapitulated early development with remarkable fidelity. Over 3-4 weeks, some of the embryoid bodies formed a bilaterally symmetric pyriform embryonic disc, an amnion, a yolk sac, and a mesoblast outgrowth attaching the caudal pole of the amnion to the culture dish.

Histological and ultrastructural examination of one of these embryoid bodies (formed from a cell line that had been passaged continuously for 6 months) revealed a remarkable resemblance to a stage 6-7 post-implantation embryo. The embryonic disc was composed of a polarized, columnar epithelial epiblast (primitive ectoderm) layer separated from a visceral endoderm (primitive endoderm) layer. Electron microscopy of the epiblast revealed apical junctional complexes, apical microvilli, subapical intermediate filaments, and a basement membrane separating the epiblast from underlying visceral endoderm. All of these elements are features of the normal embryonic disc. In the caudal third of the embryonic disc, there was a midline groove, disruption of the basement membrane, and mixing of epiblast cells with underlying endodermal cells (early primitive streak).

The amnion was composed of an inner squamous (ectoderm) layer continuous with the epiblast and an outer mesoderm layer. The bilayered yolk sac had occasional endothelial-lined spaces containing possible hematopoietic precursors.

The morphology, immortality, karyotype, and cell surface markers of these marmoset cells identify these marmoset cells as primate ES cells similar to the rhesus ES cells. Since the last cells in the mammalian embryo capable of contributing to both trophectoderm derivatives and endoderm derivatives are the totipotent cells of the early ICM, the ability of marmoset ES cells to contribute to both trophoblast and endoderm demonstrates their similarities to early totipotent embryonic cells of the intact embryo. The formation of embryoid bodies by marmoset ES cells, with remarkable structural similarities to the early post-implantation primate embryo, demonstrates the potential of marmoset ES cells to participate in complex developmental processes requiring the interaction of multiple cell types.

Given the reproductive characteristics of the common marmoset described above (efficient embryo transfer, multiple young, short generation time), marmoset ES cells will be particularly useful for the generation of transgenic primates. Although mice have provided invaluable insights into gene function and regulation, the anatomical and physiological differences between humans and mice limit the usefulness of transgenic mouse models of human diseases. Transgenic primates, in addition to providing insights into the pathogenesis of specific diseases, will provide accurate animal models to test the efficacy and safety of specific treatments.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Thomson, James

(ii) TITLE OF INVENTION:
Primate Embryonic Stem Cells

(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0,
Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTGGATTGT CTGCAGGATG GGGAA 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGAATTCGA AGCATGTCAA AGTGGTATGG 30

CLAIMS

We claim:

1. A purified preparation of primate embryonic stem cells which (i) is capable of proliferation *in vitro* culture for over one year, (ii) maintains a normal karyotype through prolonged culture, (iii) maintains the potential to differentiate to derivatives of endoderm, mesoderm, and ectoderm tissues throughout the culture, and (iv) will not differentiate when cultured on a fibroblast feeder layer.

2. The preparation of claim 1 wherein the stem cells will spontaneously differentiate to trophoblast and produce chorionic gonadotropin when cultured to high density.

3. A purified preparation of primate embryonic stem cells wherein the cells are negative for the SSEA-1 marker, positive for the SSEA-3 marker, positive for the SSEA-4 marker, express alkaline phosphatase activity, are pluripotent, and have normal karyotypes.

4. The preparation of claim 3 wherein the cells are positive for the TRA-1-60, and TRA-1-81 markers.

5. The preparation of claim 3 wherein the cells continue to proliferate in an undifferentiated state after continuous culture for at least one year.

6. The preparation of claim 3 wherein the cells will differentiate to trophoblast when cultured beyond confluence and will produce chorionic gonadotropin.

7. The preparation of claim 3 wherein the cells remain euploid for more than one year of continuous culture.

8. The preparation of claim 3 wherein the cells differentiate into cells derived from mesoderm, endoderm and ectoderm germ layers when the cells are injected into a SCID mouse.

9. A method of isolating a primate embryonic stem cell line, comprising the steps of:

(a) isolating a primate blastocyst;

(b) isolating cells from the inner cell mass of the blastocyte of (a);

(c) plating the inner cell mass cells on embryonic fibroblasts, wherein inner cell mass-derived cell masses are formed;

(d) dissociating the mass into dissociated cells;

(e) replating the dissociated cells on embryonic feeder cells;

(f) selecting colonies with compact morphologies and cells with high nucleus to cytoplasm ratios and prominent nucleoli; and

(g) culturing the cells of the selected colonies.

10. A method as claimed in claim 9 further comprising maintaining the isolated cells on a fibroblast feeder layer to prevent differentiation.

11. A cell line developed by the method of step 9.

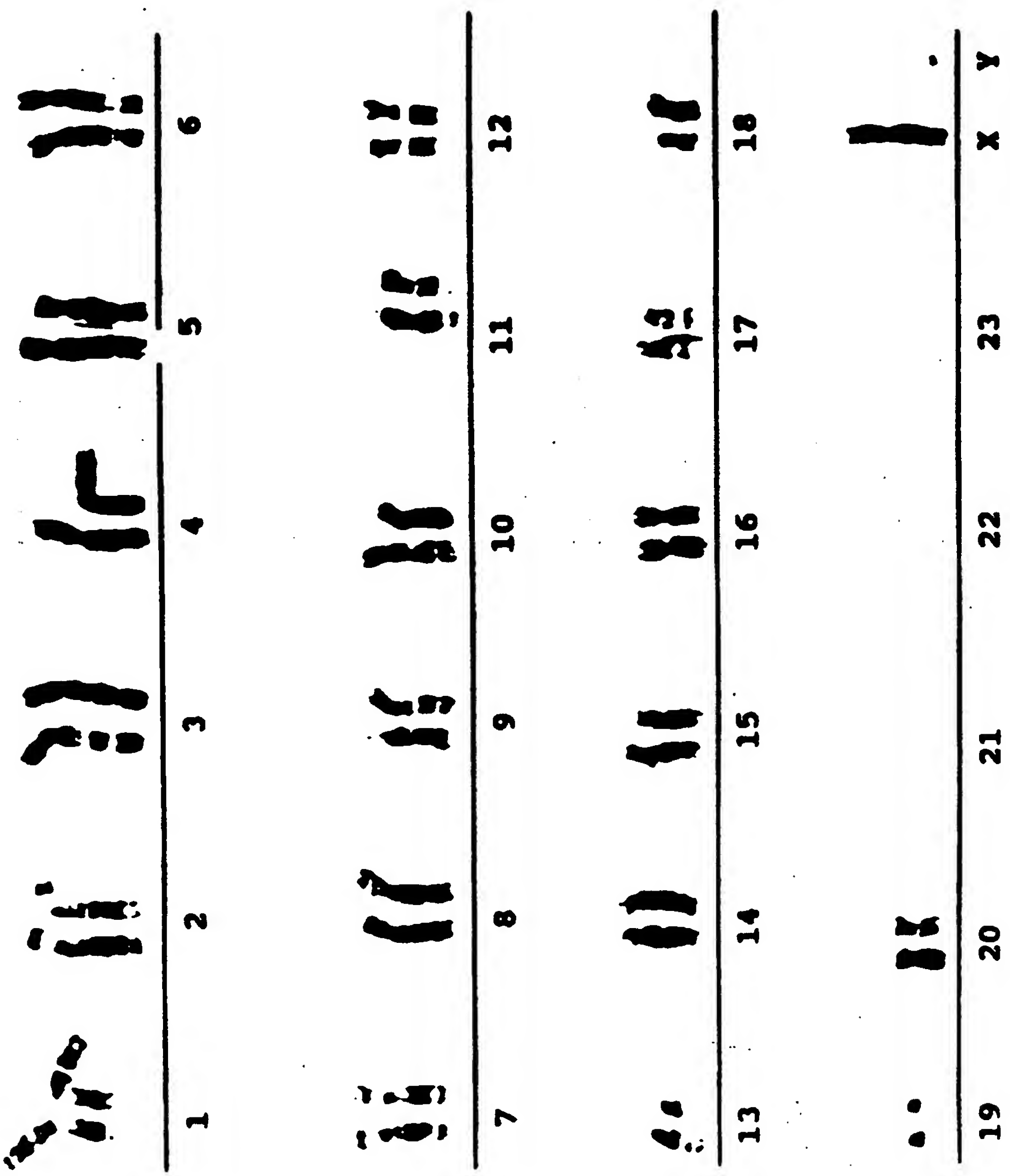

FIG. 1

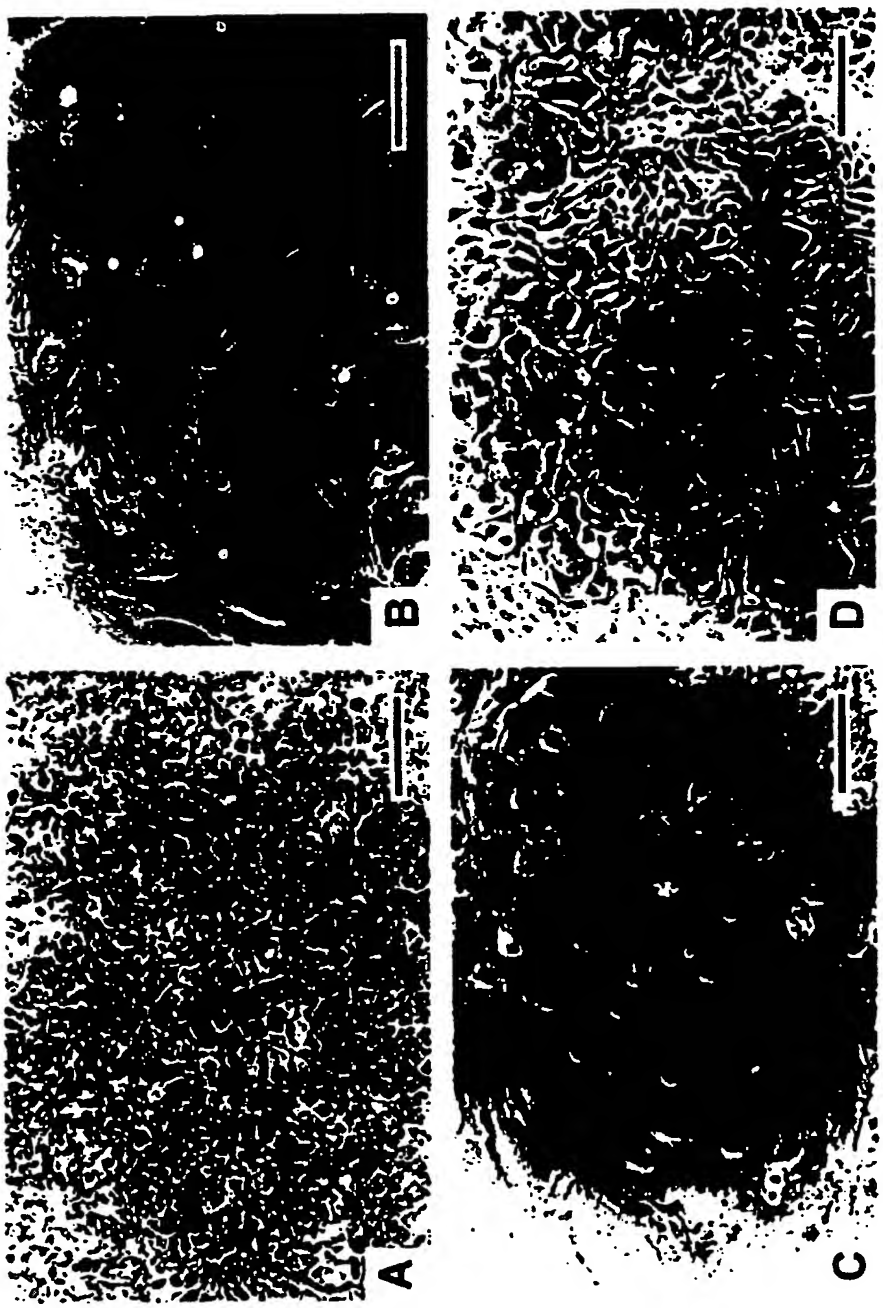

FIG 2

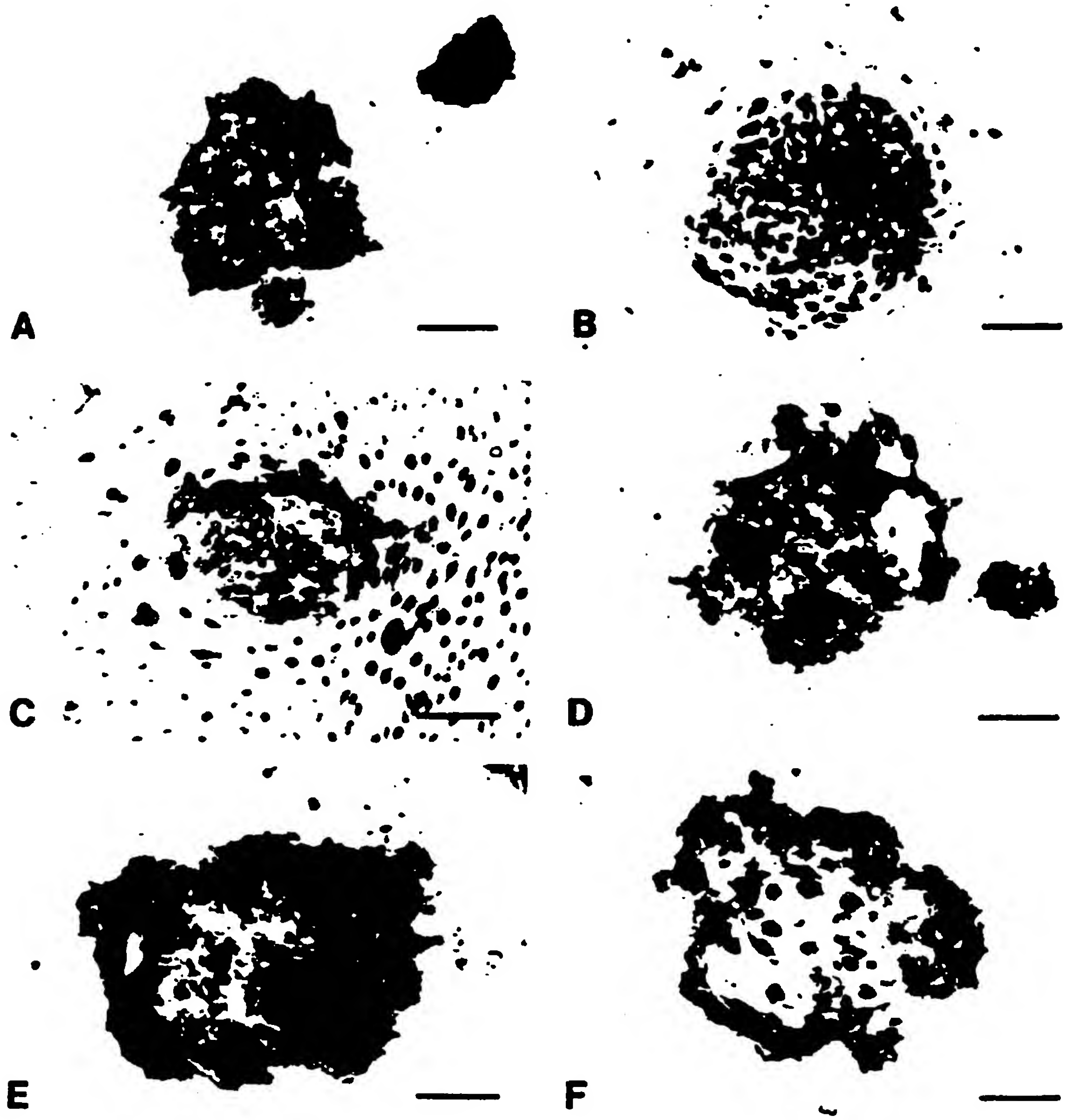

FIG. 3

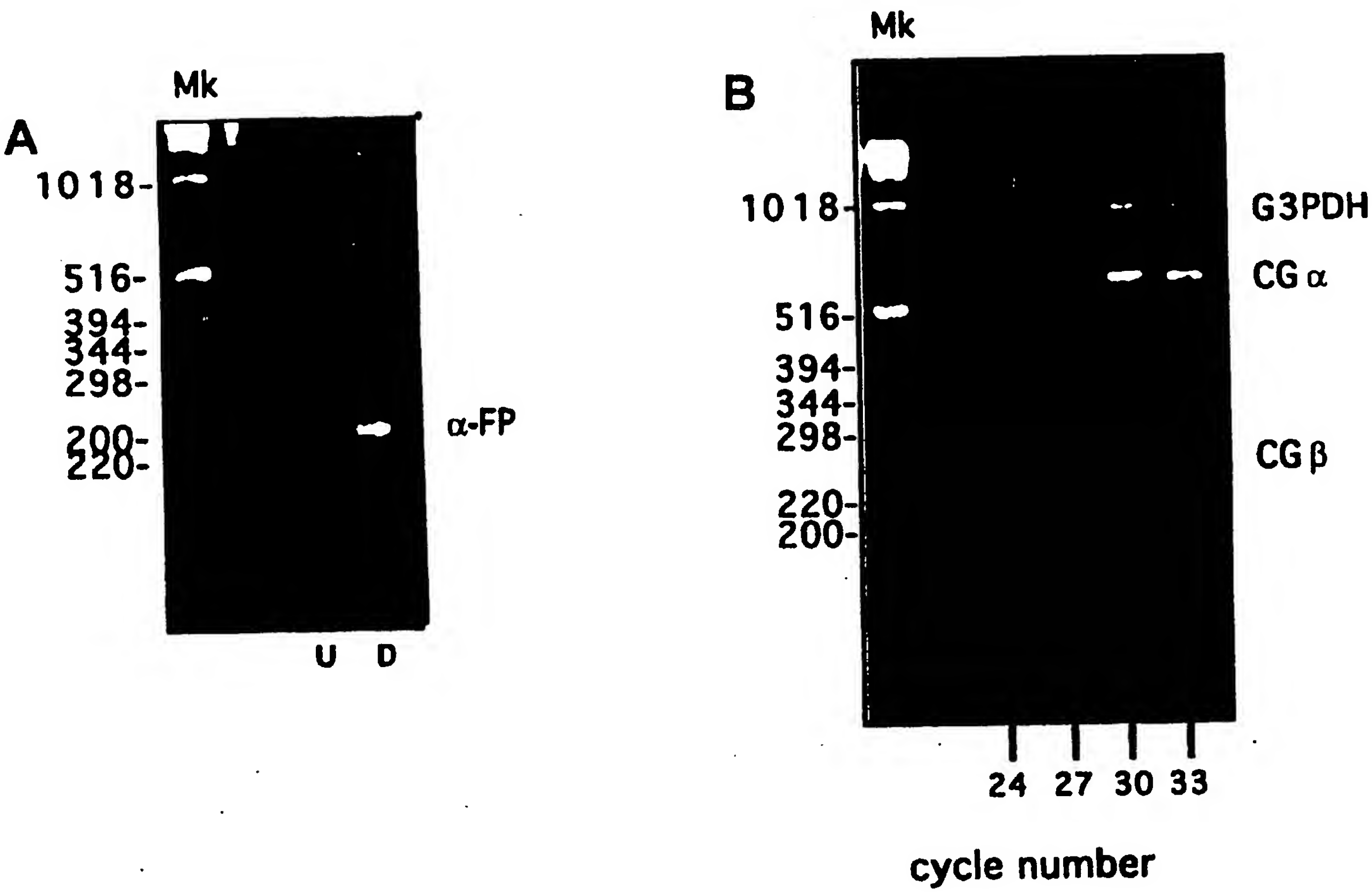

FIG. 4

Panel A. PCR amplification of α-fetoprotein cDNA from reverse transcribed total RNA from undifferentiated (U) and differentiated (D) R278.5 cells. Panel B. PCR amplification of α-chorionic gonadotropin and β-chorionic goandotropin subunit cDNAs from reverse transcribed total RNA from differentiated R278.5 cells.

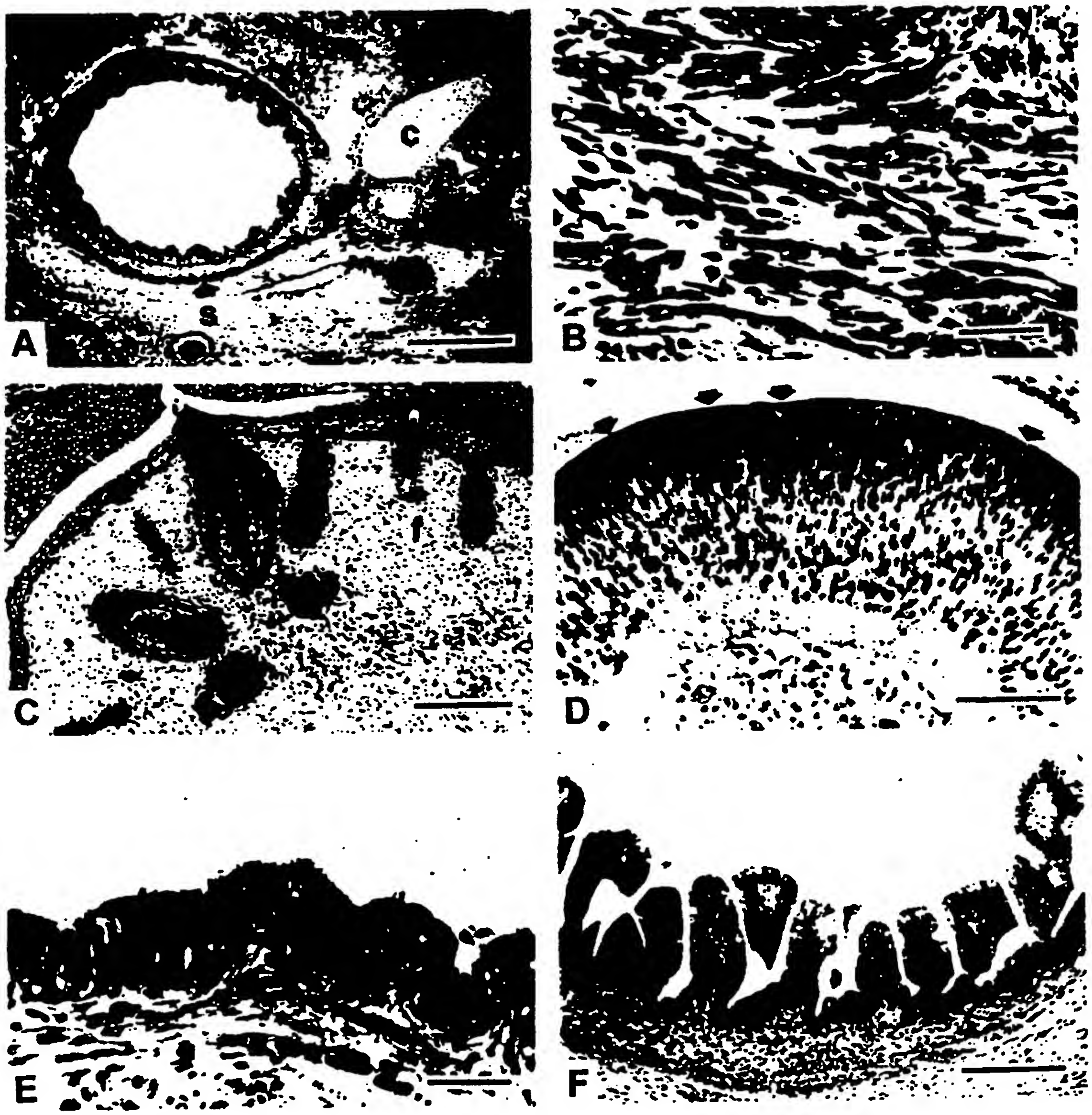

FIG. 5

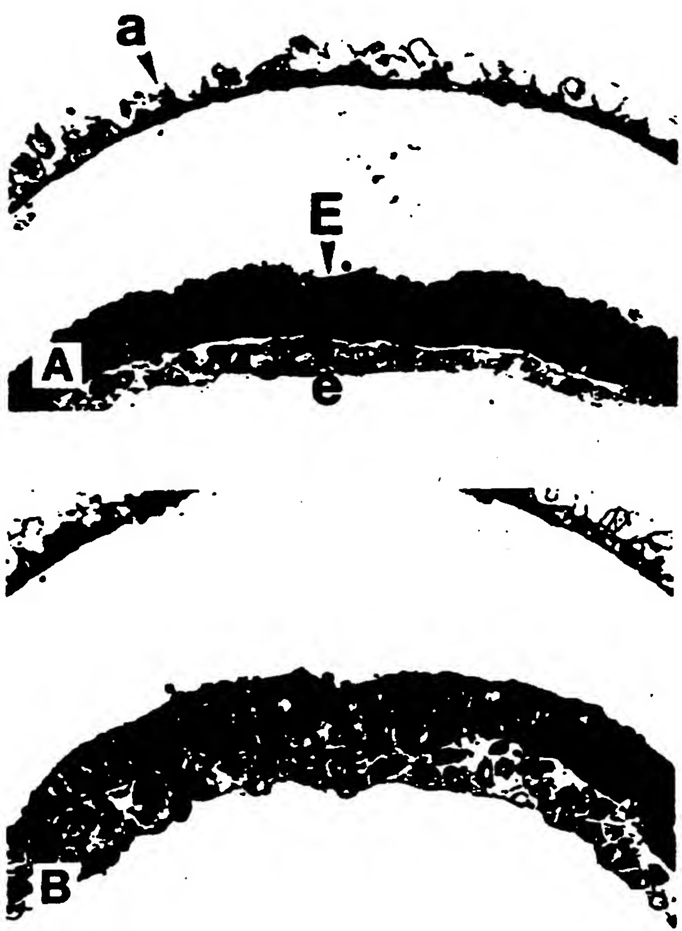

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00596

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00
US CL :435/240.2, 240.21, 240.23, 240.243, 240.3, 240.31
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2, 240.21, 240.23, 240.243, 240.3, 240.31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS
search terms: embryonic stem cells

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	The Milwaukee Journal, issued 04 November 1994, "Embryonic monkey cells isolated".	1-8 and 11
X	Theriogenology, Volume 41, issued 1994, Bongso et al., "THE GROWTH OF INNER CELL MASS CELLS FROM HUMAN BLASTOCYSTS", page 167, see entire document.	1-8 and 11
X	Human Reproduction, Volume 9, No. 11, issued 1994, Bongso et al., "Isolation and culture of inner cell mass cells from human blastocysts", page 2110-2117, see entire document.	1-8 and 11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 MARCH 1996

Date of mailing of the international search report

01 APR 1996

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00596

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Theriogenology, Volume 34, No. 5, issued november 1990, Piedrahita et al., "ON THE ISOLATION OF EMBRYONIC STEM CELLS: COMPARATIVE BEHAVIOR OF MURINE, PORCINE AND OVINE EMBRYOS", pages 879-901, see entire document.	9-11

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 13726–13731, November 1998
Developmental Biology

Derivation of pluripotent stem cells from cultured human primordial germ cells

(alkaline phosphatase/embryoid body/embryonic stem cell/embryonic germ cell)

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Contributed by John W. Littlefield, September 29, 1998

ABSTRACT **Human pluripotent stem cells would be invaluable for *in vitro* studies of aspects of human embryogenesis. With the goal of establishing pluripotent stem cell lines, gonadal ridges and mesenteries containing primordial germ cells (PGCs, 5–9 weeks postfertilization) were cultured on mouse STO fibroblast feeder layers in the presence of human recombinant leukemia inhibitory factor, human recombinant basic fibroblast growth factor, and forskolin. Initially, single PGCs in culture were visualized by alkaline phosphatase activity staining. Over a period of 7–21 days, PGCs gave rise to large multicellular colonies resembling those of mouse pluripotent stem cells termed embryonic stem and embryonic germ (EG) cells. Throughout the culture period most cells within the colonies continued to be alkaline phosphatase-positive and tested positive against a panel of five immunological markers (SSEA-1, SSEA-3, SSEA-4, TRA-1–60, and TRA-1–81) that have been used routinely to characterize embryonic stem and EG cells. The cultured cells have been continuously passaged and found to be karyotypically normal and stable. Both XX and XY cell cultures have been obtained. Immunohistochemical analysis of embryoid bodies collected from these cultures revealed a wide variety of differentiated cell types, including derivatives of all three embryonic germ layers. Based on their origin and demonstrated properties, these human PGC-derived cultures meet the criteria for pluripotent stem cells and most closely resemble EG cells.**

Pluripotent stem cells have been derived from two embryonic sources. Embryonic stem (ES) cells are derived from the inner cell mass of preimplantation embryos (1, 2), and embryonic germ (EG) cells are derived from primordial germ cells (PGCs) (3, 4). Both ES and EG cells are pluripotent and demonstrate germ-line transmission in experimentally produced chimeras (5, 6). Mouse ES and EG cells share several morphological characteristics such as high levels of intracellular alkaline phosphatase (AP), and presentation of specific cell surface glycolipids (7, 8) and glycoproteins (9). These properties are characteristic of, but not specific for, pluripotent stem cells. Other important characteristics include growth as multicellular colonies, normal and stable karyotypes, the ability to be continuously passaged, and the capability to differentiate into cells derived from all three embryonic germ layers. Pluripotent stem cell lines that share most of these characteristics also have been reported for chicken (10), mink (11), hamster (12), pig (13, 14), rhesus monkey (15), and common marmoset (16).

The pluripotency of ES and EG cells can be demonstrated *in vitro* and *in vivo*. Embryoid bodies (EBs) are differentiated cell aggregates first described as arising in human (17) and mouse (18–20) teratomas and teratocarcinomas. These aggregates range from a cluster of pluripotent stem cells enclosed by a layer of endoderm to complex structures closely resembling an embryo during early development. EBs from mouse pluripotent stem cells grown on feeder layers or in suspension may contain a variety of cell types. This property has been used as evidence of cell pluripotency (1, 21) and as a source of differentiating cells. With the proper combinations of growth and differentiation factors, mouse ES (22, 23) and EG (S.W., unpublished results) cultures can generate cells of the hematopoietic lineage and cardiomyocytes (24, 25). In addition, mouse ES cells have been used to generate *in vitro* cultures of neurons (26), skeletal muscle (27), and vascular endothelial cells (28). ES and EG cells from some species can form teratocarcinomas when injected into histocompatible or immunologically compromised mice. This property alone may not be a definitive test of stem cell pluripotency, as it has been demonstrated that rat and mouse visceral (yolk sac) endoderm are capable of forming highly differentiated teratomas containing cells of all three embryonic germ layers (29, 30). Perhaps the most definitive *in vivo* test of developmental potential would be a demonstrated contribution to all cell lineages in a chimeric animal, but this test is not practical or possible for all species and cannot be done with human cells.

We report here the establishment of cultures from human PGCs. These cultures have morphological, immunohistochemical, and karyotypic features consistent with those of previously described pluripotent stem cells and have a demonstrated ability to differentiate *in vitro* into derivatives of the three embryonic germ layers.

MATERIALS AND METHODS

Collection of Tissue, Establishment, and Maintenance of Cultures. Gonadal ridges and mesenteries of 5- to 9-week postfertilization human embryos (obtained as a result of therapeutic termination of pregnancy by using a protocol approved by the Joint Committee on Clinical Investigation of the Johns Hopkins University School of Medicine) were mechanically disaggregated then incubated in 0.05% trypsin-0.5 mM EDTA (GIBCO/BRL) or 0.25% trypsin at 37°C for 5–10 min, or incubated in a combination of 0.01% hyaluronidase type V (Sigma), 0.1% collagenase type IV (Sigma), and 0.002% DNase type I (Sigma) at 37°C for 2 hr (31). Cells initially were cultured and subsequently passaged on a mouse

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Abbreviations: AP, alkaline phosphatase; EG, embryonic germ; ES, embryonic stem; PGC, primordial germ cell; EB, embryoid body; hrLIF, human recombinant leukemia inhibitory factor; hrbFGF, human recombinant basic fibroblast growth factor.

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STO fibroblast feeder layer mitotically inactivated with 5,000 rads (1 rad = 0.01 Gy) γ-radiation. Cells were grown in DMEM (GIBCO/BRL) supplemented with 15% fetal bovine serum (HyClone), 0.1 mM nonessential amino acids (GIBCO/BRL), 0.1 mM 2-mercaptoethanol (Sigma), 2 mM glutamine (GIBCO/BRL), 1 mM sodium pyruvate (GIBCO/BRL), 100 units/ml of penicillin (GIBCO/BRL), 100 μg/ml of streptomycin (GIBCO/BRL), 1,000 units/ml of human recombinant leukemia inhibitory factor (hrLIF, Genzyme), 1 ng/ml of human recombinant basic fibroblast growth factor (hrbFGF, Genzyme), and 10 μM forskolin (Sigma). Cultures were grown in 5% or 8% CO_2, 95% humidity and were routinely passaged every 7 days after disaggregation with 0.05% trypsin/0.53 mM EDTA (GIBCO/BRL) or 0.25% trypsin at 37°C for 5–10 min.

Initial Characterization. Cells were fixed for detection of AP activity in 66% acetone/3% formaldehyde and then stained with naphthol/FRV-alkaline AP substrate (Sigma). For immunocytochemistry, cells were fixed in 3% paraformaldehyde in Dulbecco's PBS (GIBCO/BRL). Cell surface glycolipid- and glycoprotein-specific mAbs were used at 1:15 to 1:50 dilution. MC480 (SSEA-1), MC631 (SSEA-3), and MC813–70 (SSEA-4) antibodies were supplied by the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City). TRA-1–60 and TRA-1–81 were a gift of Peter Andrews (University of Sheffield, U.K.). Antibodies were detected by using biotinylated anti-mouse secondary antibody, strepavidin-conjugated horseradish peroxidase, and 3-amino-9-ethylcarbazole chromagen (BioGenex). Cells prepared for cytogenetic analysis were incubated in growth media with 0.1 μg/ml of Colcemid for 3–4 hr, trypsinized, resuspended in 0.075 M KCl, and incubated for 20 min at 37°C, then fixed in 3:1 methanol/acetic acid.

Immunohistochemistry. EBs were collected from cultures and either immediately embedded or replated into single wells of a 96-well tissue culture plate and cultured for 14 days in the absence of hrLIF, hrbFGF, and forskolin, before embedding. For embedding, EBs were placed into a small drop of molten 1% low melting point agarose (FMC) prepared in PBS, and

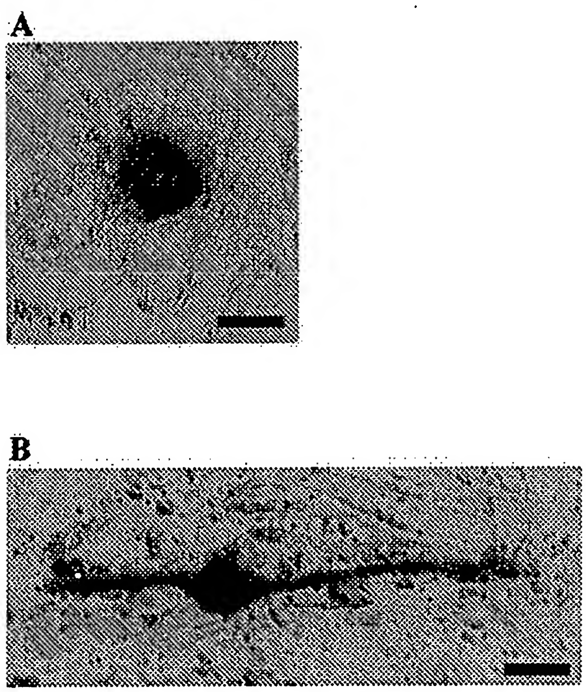

FIG. 1. AP activity of individual human PGCs in culture. (*A*) Stationary and (*B*) migratory PGCs in a primary culture, growing on a feeder layer of mitotically inactivated mouse STO fibroblasts. (Bars represent 10 μm.)

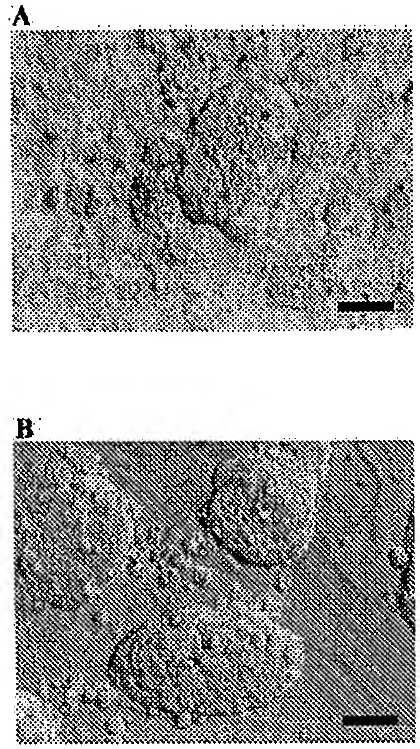

FIG. 2. Colony morphology. (*A*) Human PGC-derived cell colony growing on a feeder layer of mitotically inactivated mouse STO fibroblasts. (*B*) Mouse ES colony growing on a feeder layer of mitotically inactivated mouse fibroblasts. Hoffman modulation optics. (Bars represent 100 μm.)

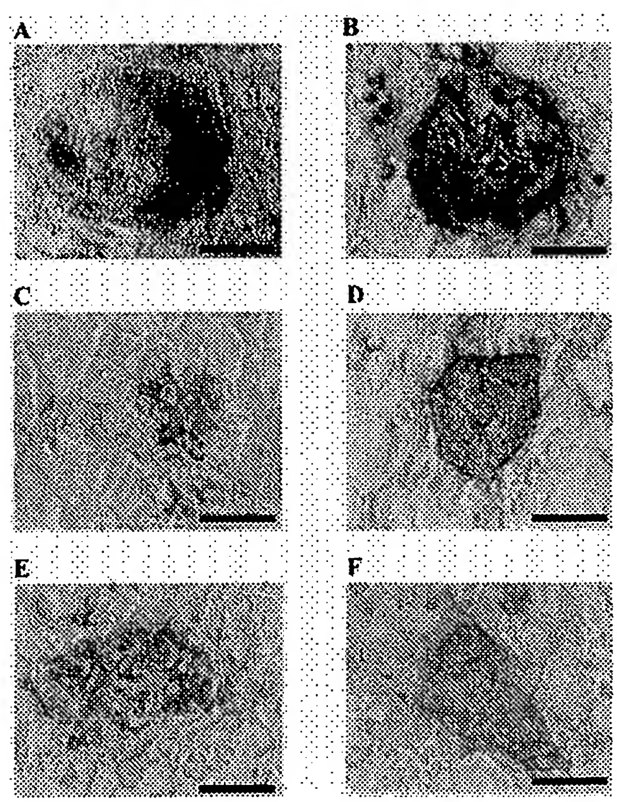

FIG. 3. Expression of cell surface markers by human PGC-derived cell colonies. (*A*) AP. (*B*) SSEA-1. (*C*) SSEA-3. (*D*) SSEA-4. (*E*) TRA-1–60. (*F*) TRA-1–81. (Bars represent 100 μm.)

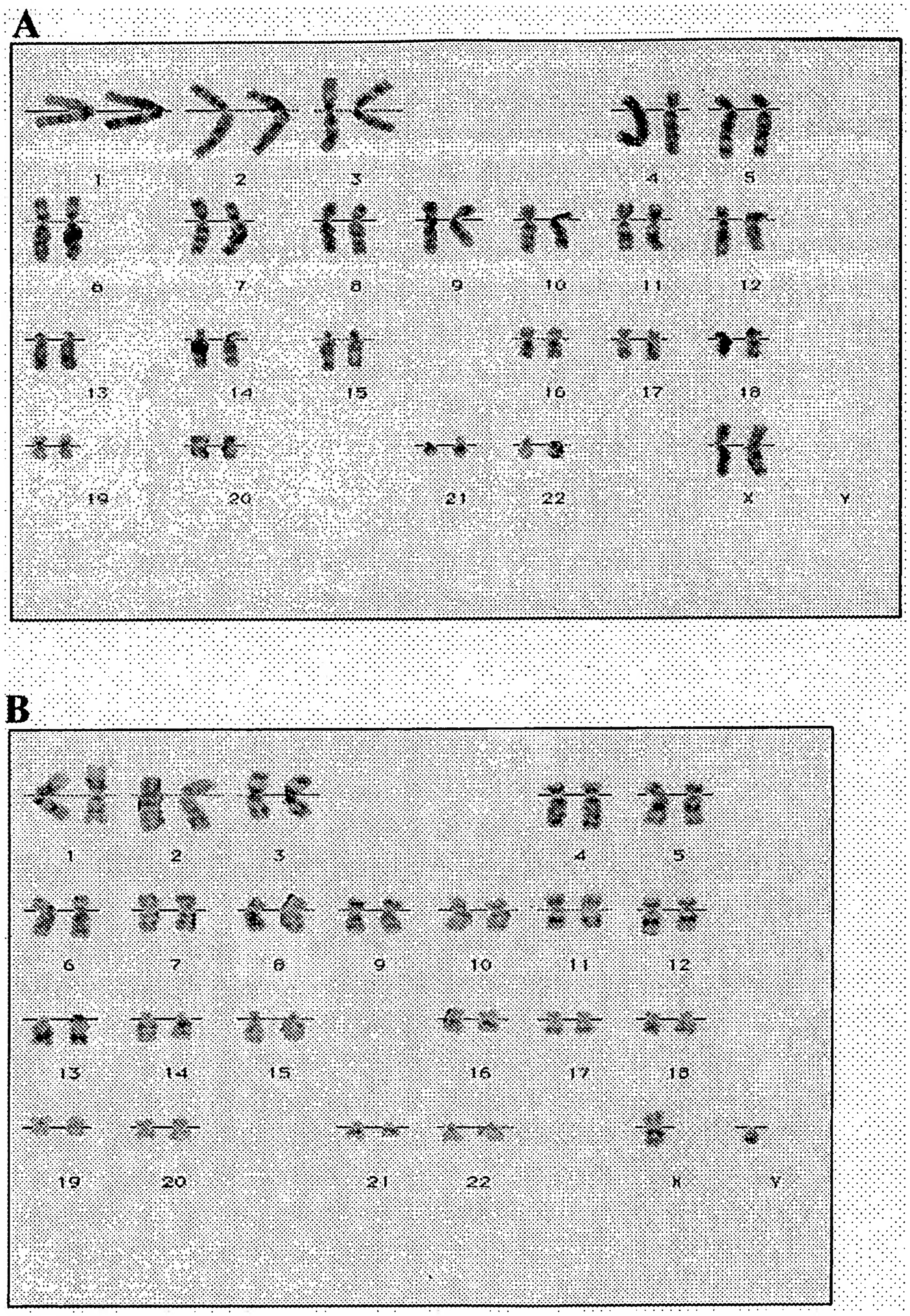

FIG. 4. Karyotype of human PGC-derived cell cultures. (*A*) XX, eight passages. (*B*) XY, 10 passages.

cooled to 42°C. Solidified agarose containing EBs then were fixed in 3% paraformaldehyde in PBS and embedded in paraffin. Individual 6-μm sections were placed on slides (ProbeOn Plus, Fisher Scientific) and immunohistochemical analysis was carried out by using a BioTek-Tech Mate 1000 automated stainer (Ventana-BioTek Solutions, Tucson, AZ). Antibodies used on paraffin sections included: HHF35 (muscle-specific actin, Dako), M 760 (desmin, Dako), CD34 (Immunotech, Luminy, France), Z 311, (S-100, Dako), sm311 (pan-neurofilament, Sternberger Monoclonals, Baltimore, MD), A 008 (alpha-1-fetoprotein, Dako), CKERAE1/AE3 (pan-cytokeratin, Boehringer Mannheim), OV-TL 12/30 (cytokeratin 7, Dako), and K_s20.8 (cytokeratin 20, Dako). Primary antibodies were detected by using biotinylated anti-rabbit

or anti-mouse secondary antibody, strepavidin-conjugated horseradish peroxidase, and diaminobenzidine chromagen (Ventana-BioTek Solutions). Slides were counterstained with hematoxylin.

RESULTS

Of 38 human PGC cultures initiated, 36 (≈95%) demonstrated morphological, biochemical, and/or immunocytochemical characteristics consistent with previously characterized pluripotent stem cell lines. High levels of AP activity are associated with ES and EG cultures, as well as with PGCs *in vivo* (32). As seen in Fig. 1, cells that closely resemble individual stationary and migratory mouse PGCs (33) are readily detected in the initial plating of human PGCs. In the presence of an irradiated STO cell feeder layer, hrbFGF, forskolin, and hrLIF, solitary PGCs gave rise to large tightly compacted multicellular colonies resembling early passage mouse ES and EG cell colonies (Fig. 2). This morphology is in contrast to the flattened and loosely associated colonies typical of human embryonal carcinoma (34) and rhesus ES cells (15). The conversion efficiency from PGCs, which have limited *in vitro* survival and proliferative capacity in the mouse (35, 36), to cells that can be passaged at least 20 times was variable and did not depend on the embryonic stage or sex of the embryo. Generation and passage of derived cultures were less successful when mouse embryo fibroblasts, human fetal fibroblasts, or gelatin-coated tissue culture dishes were substituted for STO cells, or when hrLIF or hrbFGF were withdrawn. Unlike mouse pluripotent stem cells (ES and EG), these human cells were more resistant to disaggregation by trypsin/EDTA-based reagents.

Like ES and EG cells from other species, the human PGC-derived cells possess high levels of AP activity (Fig. 3*A*). The percentage of visible AP-positive cells within a colony varied from ≈20% to >90%. Human PGC-derived cells were further characterized with a bank of five mAbs: SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (7, 8). As seen in Fig. 3 *B–F*, colonies stained strongly for four of the five antibodies, whereas colonies stained with the secondary antibody alone gave no signal (data not shown). The antibody recognizing SSEA-3 antigen stained the cells inconsistently and weakly. As with the results of AP staining, the percentage of cells within a colony that stained positive was variable.

Karyotypic analyses carried out at passage 8–10 (60–70 days in culture) indicated apparently normal human chromosomes at the 300 band level of resolution (37). Of five different cultures examined, three were XX and two were XY. Karyotypes of each sex are shown in Fig. 4.

In the human PGC-derived cultures, a small percentage (≈1–20%) of colonies spontaneously generate EBs in the presence of hrLIF. Table 1 indicates antibody reactivity of the EBs. Immunohistochemical analysis of serial sections represents a sampling of the cells within an EB so the absence of a particular marker is not significant. However, most of the markers were represented in all of the EBs.

Table 1. Immunohistochemical analysis of six human embryoid bodies

		Embryoid body					
Epitope	Marker	BF1	KF1	RI3	RI4	RI5	BF2
Muscle actin	Mesoderm	+	+	+	+	+	+
Desmin		+	+	+	ND	+	+
CD34		+	+	−	+	−	−
S-100	Ectoderm	+	+	+	−	+	+
Neurofilament, ps		ND	+	−	+	+	+
Alpha fetoprotein	Endoderm	+	+	+	+	+	+
Cytokeratin, ps	+	+	+	+	+	+	
Cytokeratin 7		+	+	ND	ND	+	+

ND, not determined because of tissue loss. +, − indicate positive and negative antibody reactivity, respectively. ps, pan-specific antibody reactivity. Embryoid bodies were formed in the presence of hrLIF, hrbFGF, and forskolin. RI3, RI4, RI5, and BF2 were subsequently cultured 2 weeks in the absence of these factors.

Immunohistochemical analysis of the EBs demonstrated that PGC-derived cells can differentiate into a variety of cell types, including derivatives of the three embryonic germ layers. Three distinct mesodermal derivatives were seen. These were antimuscle specific actin-reactive myocytes with prominent eccentric nuclei and cytoplasmic filaments (Fig. 5*A*), anti-desmin-reactive mesenchymal cells (Fig. 5*B*), and anti-CD34-reactive vascular endothelium (Fig. 5*C*). Ectodermal derivatives include cells suggestive of neuroepithelia with nuclear localized anti-S-100-reactivity (Fig. 5 *D* and *E*) and antineurofilament-reactive cells (Fig. 5*F*). Endodermal derivatives include anti-α-1-fetoprotein-reactive cells, which appear within the interior of some EBs as well as form the exterior layer (Fig. 5*G*). Several types of anticytokeratin-reactive epithelia were seen, including nests of relatively undifferentiated cells (Fig. 5*H*) and simple cuboidal epithelial layers (Fig. 5*I*).

DISCUSSION

The human cultures described here satisfy the criteria used to define pluripotent stem cells. These include presentation of a series of markers commonly used to identify pluripotent stem cells, morphological similarity to mouse ES and EG cells, normal and stable karyotype maintained over at least 10 passages, and demonstrated ability to differentiate into a wide variety of cell types.

The histological profile of these human cells (AP+, SSEA-1+, SSEA-3+, SSEA-4+, TRA-1-60+, and TRA-1-81+) differs from undifferentiated human embryonal carcinoma (EC) and rhesus ES cells, which are SSEA-1 negative (15, 38). The fact that differentiation of the human EC line NTERA2 leads to increased expression of SSEA-1 may suggest that this marker is indicative of differentiation in the human PGC-derived cultures. However, NTERA2 differentiation is accompanied by the loss of the other markers (39, 40), which we do not observe. A second possibility is that SSEA-1 reactivity reflects an intrinsic difference between the relatively flat and loosely associated human EC and rhesus ES colonies and the multilayered and tightly compacted colonies formed by mouse ES and EG cells that are SSEA-1 positive.

Human cell cultures derived and grown as described have been continuously maintained for more than 20 passages. Maintaining high colony density and derivation of clonal cell lines is complicated by the difficulties associated with disaggregation of colonies to single cells. The highly compacted nature of these colonies suggests strong cell–cell adhesion. These interactions are notably more resistant to trypsin than mouse ES and EG colonies. Alternative disaggregation enzymes are currently under investigation.

The most useful and important property of these cells is their ability to differentiate *in vitro* into ectodermal, endodermal, and mesodermal derivatives. As with mouse ES and EG cells, human pluripotent stem cells can form EBs. These structures appear to recapitulate the normal developmental processes of early embryonic stages and promote the cell–cell interaction required for cell differentiation. Typically, mouse EBs are surrounded by a layer of visceral or parietal endoderm and contain a heterogeneous mixture of cell types. Morphologically, human EBs resemble those generated from mouse ES and EG cultures. Many have an outer layer that stains positive for α-1-fetoprotein (Fig. 5*G*) although the constituent cells of this layer do not always resemble those seen in mouse EBs. Identification of cell derivatives of the three embryonic germ layers in human EBs suggests that these cells are pluripotent and are capable of *in vitro* differentiation.

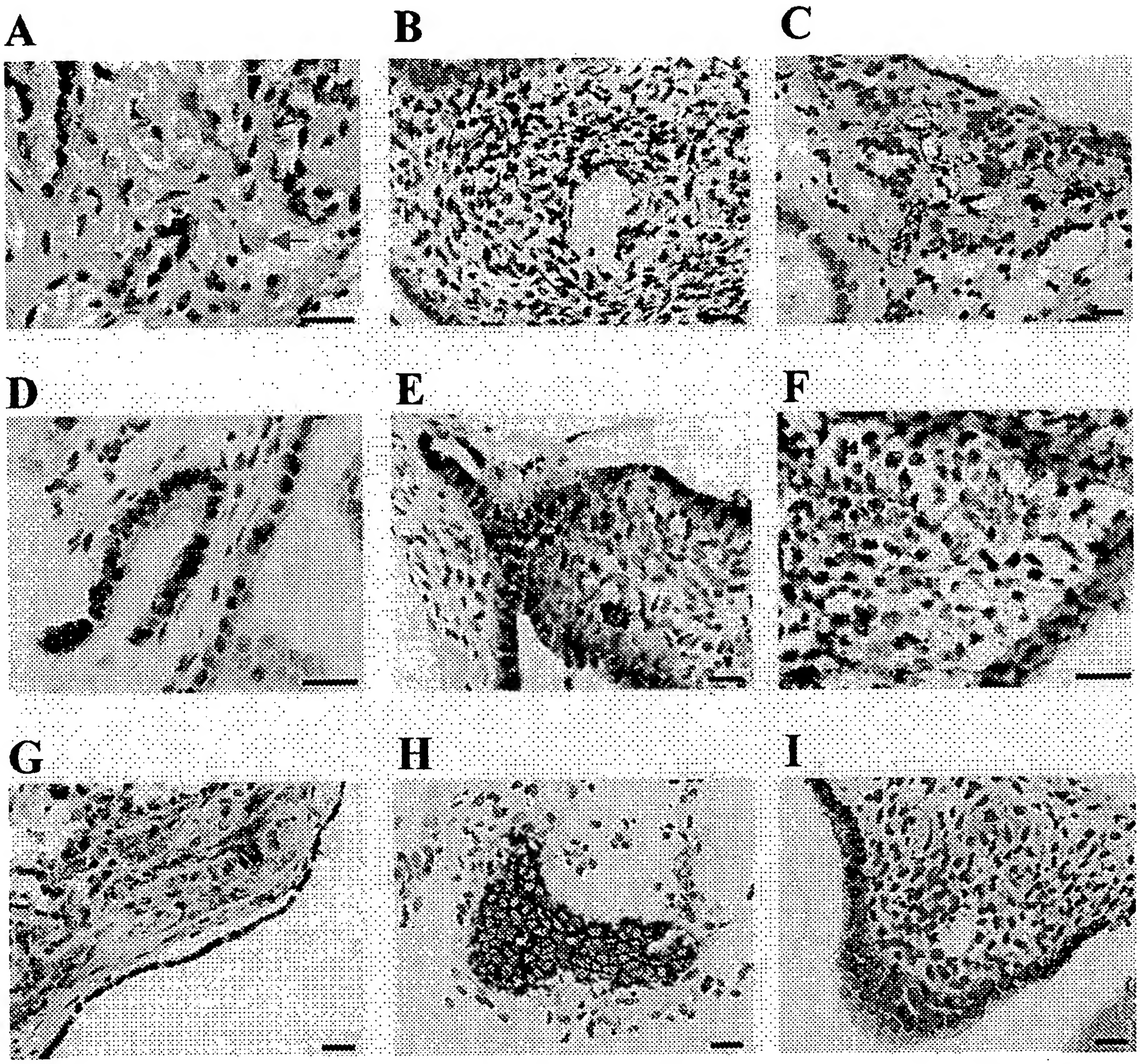

FIG. 5. Immunohistological analysis of human EB sections. EB culture designation, antibody epitope, and objective power are as follows: (*A*) BF1, muscle-specific actin, ×100. Arrow indicates a cell with eccentric nuclei and cytoplasmic filaments. (*B*) RI5, desmin, ×60. (*C*) BF1, CD34, ×60. (*D*) BF1, S-100, ×100. (*E*) RI5, S-100, ×60. (*F*) RI, pan-neurofilament, ×100. (*G*) BF1, α-1-fetoprotein, ×60. (*H*) BF1, pan-cytokeratin, ×60. (*I*) RI5, cytokeratin 7, ×60. (Bars represent 20 μm.)

Although the properties of the cultures described in this paper are consistent with those of pluripotent stem cells, the cultures have a lower plating efficiency than most mouse EG and ES cell cultures, which may reflect difficulties associated with complete cell disaggregation. Although many of the PGC-derived cultures have been passaged 20–25 times, the immortality of these cells remains to be demonstrated.

Human pluripotent stem cells, with their potential to differentiate into a wide variety of cell types in culture, would be invaluable for studies of some aspects of human embryogenesis and for transplantation therapies. They may serve to define the culture conditions and differential gene expression necessary for cell-type-specific differentiation and for the isolation of lineage-restricted stem cells that could serve as a source of cells for transplantation. Genetic modification of these pluripotent stem cells may allow the generation of universal donor cells or cells that have been customized to meet individual requirements. Clearly, these goals warrant investigations on the isolation, study, and use of human pluripotent stem cells.

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1. Evans, M. J. & Kaufman, M. H. (1981) *Nature (London)* **292,** 154–156.
2. Martin, G. R. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7634–7638.
3. Matsui, Y., Toksoz, D., Nishikawa, S., Nishikawa, S., Williams, D., Zsebo, K. & Hogan, B. L. (1991) *Nature (London)* **353,** 750–752.
4. Resnick, J. L., Bixler, L. S., Cheng, L. & Donovan, P. J. (1992) *Nature (London)* **359,** 550–551.
5. Stewart, C., Gadi, I. & Bhatt, H. (1994) *Dev. Biol.* **161,** 626–628.
6. Labosky, P., Barlow, D. & Hogan, B. (1994) *Development (Cambridge, U.K.)* **120,** 3197–3204.
7. Solter, D. & Knowles, B. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5565–5569.
8. Kannagi, R., Cochran, N., Ishigami, F., Hakomori, S., Andrews, P., Knowles, B. & Solter, D. (1983) *EMBO J.* **2,** 2355–2361.

9. Andrews, P., Banting, G., Damjanov, I., Arnaud, D. & Avner, P. (1984) *Hybridoma* **3**, 347–361.
10. Pain, B., Clark, M. E., Shen, M., Nakazawa, H., Sakurai, M., Samarut, J. & Etches, R. J. (1996) *Development (Cambridge, U.K.)* **122**, 2339–2348.
11. Sukoyan, M. A., Vatolin, S. Y., Golubitsa, A. N., Zhelezova, A. I., Semenova, L. A. & Serov, O. L. (1993) *Mol. Reprod. Dev.* **36**, 148–158.
12. Doetschman, T., Williams, P. & Maeda, N. (1988) *Dev. Biol.* **127**, 224–227.
13. Wheeler, M. B. (1994) *Reprod. Fertil. Dev.* **6**, 563–568.
14. Shim, H., Gutierrez-Adan, A., Chen, L., BonDurant, R., Behboodi, E. & Anderson, G. (1997) *Biol. Reprod.* **57**, 1089–1095.
15. Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P., Becker, R. A. & Hearn, J. P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 7844–7848.
16. Thomson, J. A., Kalishman, J., Golos, T. G., Durning, M., Harris, C. P. & Hearn, J. P. (1996) *Biol. Reprod.* **55**, 254–259.
17. Peyron, A. (1939) *Bull. Assoc. franç. étude cancer* **28**, 658–681.
18. Stevens, L. C. (1958) *J. Natl. Cancer Inst.* **20**, 1257–1270.
19. Stevens, L. C. (1959) *J. Natl. Cancer Inst.* **23**, 1249–1295.
20. Stevens, L. C. (1970) *Dev. Biol.* **21**, 364–382.
21. Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W. & Kemler, R. (1985) *J. Embryol. Exp. Morphol.* **87**, 27–45.
22. Wiles, M. V. & Keller, G. (1991) *Development (Cambridge, U.K.)* **111**, 259–267.
23. Keller, G., Kennedy, M., Papayannopoulou, T. & Wiles, M. V. (1993) *Mol. Cell Biol.* **13**, 473–486.
24. Klug, M., Soonpaa, M. & Field, L. (1995) *Am. J. Physiol.* **269**, H1913–H1921.
25. Rohwedel, J., Sehlmeyer, U., Shan, J., Meister, A. & Wobus, A. (1996) *Cell. Biol. Int.* **20**, 579–587.
26. Bain, G., Kitchens, D., Yao, M., Huettner, J. & Gottlieb, D. (1995) *Dev. Biol.* **168**, 342–357.
27. Rohwedel, J., Maltsev, V., Bober, E., Arnold, H.-H., Hescheler, J. & Wobus, A. (1994) *Dev. Biol.* **164**, 87–101.
28. Wang, R., Clark, R. & Bautch, V. (1992) *Development (Cambridge, U.K.)* **114**, 303–316.
29. Sobis, H., Verstuyf, A. & Vandeputte, M. (1991) *Development (Cambridge, U.K.)* **111**, 75–78.
30. Sobis, H., Verstuyf, A. & Vandeputte, M. (1993) *Int. J. Dev. Biol.* **37**, 155–168.
31. Belldegrun, A., Kasid, A., Uppenkamp, M., Topalian, S. & Rosenberg, S. (1989) *J. Immunol.* **142**, 4520–4526.
32. Chiquoine, A. D. (1954) *Anat. Rec.* **118**, 135–146.
33. Donovan, P. J., Stott, D., Cairns, L. A., Heasman, J. & Wylie, C. C. (1986) *Cell* **44**, 831–838.
34. Andrews, P. W., Bronson, D. L., Benham, F., Strickland, S. & Knowles, B. B. (1980) *Int. J. Cancer* **26**, 269–280.
35. Dolci, S., Williams, D. E., Ernst, M. K., Resnick, J. L., Brannan, C. I., Lock, L. F., Lyman, S. D., Boswell, H. S. & Donovan, P. J. (1991) *Nature (London)* **352**, 809–811.
36. Matsui, Y., Zsebo, K. & Hogan, B. L. (1992) *Cell* **70**, 841–847.
37. Mitelman, F. (1995) *An International System for Human Cytogenetic Nomenclature* (Karger, Basel).
38. Wenk, J., Andrews, P. W., Casper, J., Hata, J., Pera, M. F., von Keitz, A., Damjanov, I. & Fenderson, B. A. (1994) *Int. J. Cancer* **58**, 108–115.
39. Andrews, P. W., Goodfellow, P. N., Shevinsky, L. H., Bronson, D. L. & Knowles, B. B. (1982) *Int. J. Cancer* **29**, 523–531.
40. Damjanov, I., Fox, N., Knowles, B. B., Solter, D., Lange, P. H. & Fraley, E. E. (1982) *Am. J. Pathol.* **108**, 225–230.

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Isolation of a primate embryonic stem cell line

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ABSTRACT **Embryonic stem cells have the ability to remain undifferentiated and proliferate indefinitely *in vitro* while maintaining the potential to differentiate into derivatives of all three embryonic germ layers. Here we report the derivation of a cloned cell line (R278.5) from a rhesus monkey blastocyst that remains undifferentiated in continuous passage for >1 year, maintains a normal XY karyotype, and expresses the cell surface markers (alkaline phosphatase, stage-specific embryonic antigen 3, stage-specific embryonic antigen 4, TRA-1-60, and TRA-1-81) that are characteristic of human embryonal carcinoma cells. R278.5 cells remain undifferentiated when grown on mouse embryonic fibroblast feeder layers but differentiate or die in the absence of fibroblasts, despite the presence of recombinant human leukemia inhibitory factor. R278.5 cells allowed to differentiate *in vitro* secrete bioactive chorionic gonadotropin into the medium, express chorionic gonadotropin α- and β-subunit mRNAs, and express α-fetoprotein mRNA, indicating trophoblast and endoderm differentiation. When injected into severe combined immunodeficient mice, R278.5 cells consistently differentiate into derivatives of all three embryonic germ layers. These results define R278.5 cells as an embryonic stem cell line, to our knowledge, the first to be derived from any primate species.**

Embryonic stem (ES) cells, derived from preimplantation embryos (1, 2), and embryonic germ (EG) cells, derived from fetal germ cells (3, 4), are undifferentiated, immortal cells capable of differentiating into derivatives of all three embryonic germ layers. Well-characterized ES and EG cells have been derived only from rodents (1, 2, 5, 6). Pluripotent cell lines have been derived from preimplantation embryos of several non-rodent species (7–10), but the developmental potentials of these cell lines remain poorly characterized. Mouse ES cells remain undifferentiated through serial passages when cultured in the presence of leukemia inhibitory factor (LIF) and differentiate in the absence of LIF (11). Mouse ES cells injected into syngeneic mice form teratocarcinomas that exhibit disorganized differentiation, with representatives of all three embryonic germ layers. Mouse ES cells combined with normal preimplantation embryos as chimeras and returned to the uterus participate in normal development (12). Because mouse ES cells can contribute to functional germ cells in chimeras, specific genetic changes can be introduced into the mouse germ line through the use of ES cell chimeras (13).

The mechanisms controlling differentiation of specific lineages can be studied with mouse ES cells grown *in vitro*; however, significant differences between early human and mouse development suggest that human development will be more accurately represented by primate ES cells. For example, human and mouse embryos differ in the timing of embryonic genome expression (14), in the structure and function of the fetal membranes and placenta (15), and in formation of an embryonic disc instead of an egg cylinder. Human embryonal carcinoma (EC) cells, which are pluripotent, immortal stem cells from teratocarcinomas, provide an important *in vitro* model for understanding human differentiation (16). Some EC cell lines can be induced to differentiate in culture (17), which results in the loss of specific cell surface markers [stage-specific embryonic antigen 3 (SSEA-3), SSEA-4, TRA-1-60, and TRA-1-81] and the appearance of new markers (16). When pluripotent human EC cells are injected into immunocompromised mice, they form teratocarcinomas, some with derivatives of all three embryonic germ layers. However, there are limitations to the use of human EC cells in the study of development. (*i*) The range of differentiation obtained from human EC cell lines is more limited than that obtained from mouse ES cells and varies widely between cell lines (18). (*ii*) All pluripotent human EC cell lines derived to date are aneuploid (19), suggesting EC cells may not provide a completely accurate representation of normal differentiation. (*iii*) Ethical considerations severely restrict the study of human embryos, often making it impossible to verify that *in vitro* results have significance in the intact embryo. None of these limitations would be present with nonhuman primate ES cell lines.

Here we report the isolation of an ES cell line (R278.5) from a rhesus monkey blastocyst. This cloned cell line remains undifferentiated and continues to proliferate for >1 year in culture, maintains a normal XY karyotype, and maintains the potential to differentiate into trophoblast and to derivatives of embryonic endoderm, mesoderm, and ectoderm. The morphology, cell surface markers, and growth factor requirements of these cells differ significantly from mouse ES cells but closely resemble human EC cells.

MATERIALS AND METHODS

Cell Line Isolation. Six days after ovulation, an azonal blastocyst was recovered by a nonsurgical uterine flush technique from a 15-year-old rhesus monkey (20). The trophectoderm was removed by immunosurgery (21) using a rabbit anti-rhesus spleen cell antiserum followed by exposure to guinea pig complement. The intact inner cell mass (ICM) was separated from lysed trophectoderm cells and plated on mouse embryonic fibroblasts [previously exposed to 3000 rads (1 rad = 0.01 Gy) γ-radiation] in medium consisting of 80% Dulbecco's modified Eagle medium (4500 mg of glucose per liter, with L-glutamine, without sodium pyruvate; GIBCO) with 20% fetal bovine serum (HyClone), 0.1 mM 2-mercaptoethanol (Sigma), 1% nonessential amino acid stock (GIBCO) (22), and 1000 units of cloned human LIF per ml (GIBCO). After 16 days of culture, a central mass of cells was removed from

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Abbreviations: CG, chorionic gonadotropin; ES, embryonic stem; EC, embryonal carcinoma; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ICM, inner cell mass; LIF, leukemia inhibitory factor; RT-PCR, reverse transcription polymerase chain reaction; SCID, severe combined immunodeficiency; SSEA, stage-specific embryonic antigen.

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epithelial outgrowths, exposed for 3 min to 0.05% trypsin-EDTA (GIBCO), gently dissociated by pipetting through a micropipette, and replated on mouse embryonic fibroblasts. After 3 weeks of growth, colonies with a morphology resembling human EC cells were selected and expanded. At five passages, individual cells were selected by micropipette and plated in individual wells of a 96-well plate (Falcon) with mouse embryonic fibroblast feeder layers. One clone with a normal karyotype (R278.5) was expanded for further analysis.

Cell Surface Markers. R278.5 cells grown on a layer of mouse embryonic fibroblasts were used to examine the expression of cell surface markers. Alkaline phosphatase was detected histochemically following fixation of cells with 100% ethanol using "Vector red" (Vector Laboratories) as a substrate, as described by the manufacturer. The SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 antigens were detected by immunocytochemistry with specific primary monoclonal antibodies (gifts of Peter Andrews, University of Sheffield, U.K.) (16, 23–25) and localized with a biotinylated secondary antibody and then an avidin/biotinylated horseradish peroxidase complex (Vectastain ABC system, Vector Laboratories).

***In Vitro* Differentiation.** R278.5 cells were plated at low density (~5000 cells/cm^2 of surface area) in the absence of fibroblasts on gelatin-treated four-well tissue culture plates (Nunc) in the same medium as that used for initial cell line isolation, but with 0–10^4 units of added human LIF per ml (GIBCO). The resulting differentiated cells were photographed 8 days after plating.

A mouse Leydig cell bioassay (26) was used to measure luteinizing hormone/chorionic gonadotropin (CG) activity in medium conditioned for 2 days either by undifferentiated R278.5 cells (at 80% confluence on fibroblast feeder layers) or by spontaneously differentiated R278.5 cells (cultured for 2 weeks after achieving confluence on fibroblast feeders). The relative levels of the mRNAs for α-fetoprotein and the α- and β-subunits of CG relative to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were determined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) (27) using RNA from the same undifferentiated and differentiated cells. The PCR primers for human G3PDH (Clontech) do not amplify mouse G3PDH mRNA. Primers for human α-fetoprotein mRNA flank the seventh intron (5′ primer, 5′-GCTGGATTGTCTGCAGGATGGGGAA; 3′ primer, 5′-TCCCCTGAAGAAAATTGGTTAAAAT) and amplify a cDNA of 216 bp. Primers for the β-subunit of human CG flank the second intron (5′ primer, 5′-*ggatc*CACCGTCAACACCACCATCTGTGC; 3′ primer, 5′-*ggatc*CACAGGTCAAAGGGTGGTCCTTGGG) (nucleotides added to the CGβ sequence to facilitate subcloning are shown in italics) and amplify a cDNA of 262 bp. The primers for the CGα subunit were based on sequences of the first and fourth exon of the rhesus gene (28) (5′ primer, 5′-*gggaattc*GCAGTTACTGAGAACTCACAAG; 3′ primer, 5′-*gggaattc*GAAGCATGTCAAAGTGGTATGG) and amplify a cDNA of 556 bp. The identity of all cDNAs was verified by sequencing (not shown).

For RT-PCR, 1–5 μl of total R278.5 RNA was reverse transcribed, and 1–20 μl of reverse transcription reaction was subjected to the PCR in the presence of 2.5 μCi of deoxycytidine 5′-[α-^{32}P]triphosphate (1 Ci = 37 GBq; DuPont). The number of amplification rounds that produced linear increases in target cDNAs and the relation between input RNA and amount of PCR product were empirically determined. Following agarose gel electrophoresis, DNA bands of interest were cut out and radioactivity was determined by liquid scintillation spectroscopy. The ratio of cpm in a specific PCR product relative to cpm of G3PDH PCR product was used to estimate the relative levels of mRNAs among differentiated and undifferentiated cells.

Tumor Formation in Severe Combined Immunodeficient (SCID) Mice. In the passage immediately prior to SCID mouse injection (7 months after initial derivation of R278), karyotypes of R278.5 were confirmed as euploid. Approximately 5 $\times$ 10^5 R278.5 cells were injected either into the rear leg muscles (seven mice) or into the testis (two mice) of 8- to 12-week-old male SCID mice. The resulting tumors were fixed in 4% paraformaldehyde and examined histologically after paraffin embedding at 8–15 weeks of development.

RESULTS

The morphology and cell surface markers of R278.5 cells (Fig. 1*A*) more closely resembled human EC cells than mouse ES cells. R278.5 cells had a high nucleus/cytoplasm ratio and prominent nucleoli, but rather than forming compact, piled-up colonies with indistinct cell borders similar to mouse ES cells, R278.5 cells formed flatter colonies with individual, distinct cells. R278.5 cells expressed alkaline phosphatase activity and the cell surface antigens SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (Fig. 2), cell surface markers characteristic of human EC cell lines (16). Although cloned human LIF was present in the medium at cell line derivation and for initial passages, R278.5 cells grown on mouse embryonic fibroblasts without exogenous LIF remained undifferentiated and continued to proliferate. R278.5 cells plated on gelatin-treated tissue culture plates without fibroblasts differentiated to multiple cell types or failed to attach and died, regardless of the presence or absence of exogenously added human LIF (Fig. 1*B*).

The mRNA for α-fetoprotein, a marker for endoderm, increased substantially with *in vitro* differentiation (Fig. 3). α-Fetoprotein is expressed by extra-embryonic (yolk sac) and embryonic (fetal liver and intestines) endoderm. Epithelial cells resembling extraembryonic endoderm were present in cells differentiated *in vitro* from R278.5 cells (Fig. 1*B*).

Luteinizing hormone activity, an indication of CG secretion and trophoblast differentiation, was present in culture medium collected from differentiated cells [3.89 milli-international units (mIU)/ml] but not in medium collected from undiffer-

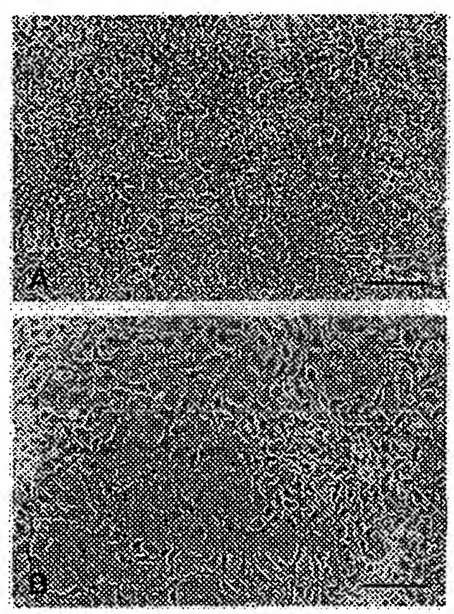

FIG. 1. Colony morphology and *in vitro* differentiation of cell line R278.5. (*A*) Undifferentiated R278.5 cells. Note the distinct cell borders, high nucleus to cytoplasm ratio, and prominent nucleoli. (Bar = 100 μm.) (*B*) Differentiated cells 8 days after plating R278.5 cells on gelatin-treated tissue culture plastic, with 10^3 units of added human LIF per ml. (Bar = 100 μm.)

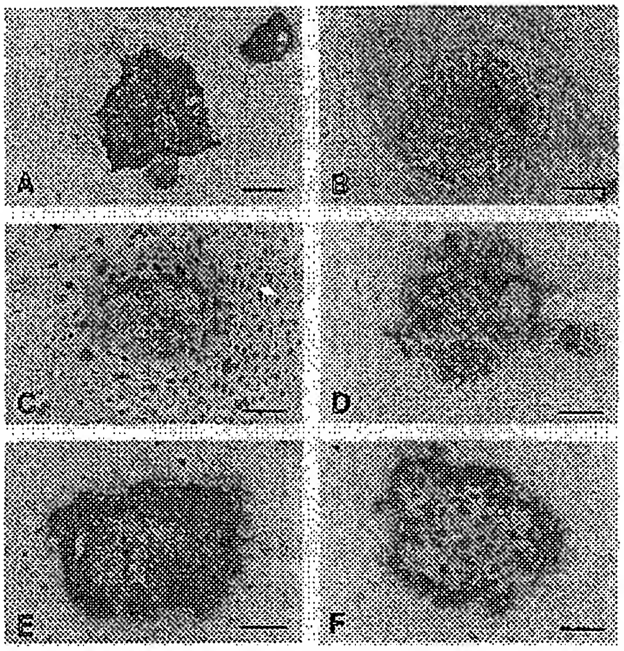

FIG. 2. Expression of cell surface markers by undifferentiated R278.5 cells. (*A*) Alkaline phosphatase. (*B*) SSEA-1. (*C*) SSEA-3. (*D*) SSEA-4. (*E*) TRA-1-60. (*F*) TRA-1-81. (Bars = 100 μm.) SSEA-3 staining of R278.5 cells was consistently weaker than the other positive antigens, and cell staining intensity varied within and between colonies.

entiated cells (<0.03 mIU/ml). The mRNAs for the CG subunits were readily detectable in the differentiated cells, although the relative level of the CGβ subunit mRNA was considerably lower than that for the CGα subunit (Fig. 4). The relative level of the CGα mRNA was quite low in undifferentiated cells, but the relative level was increased 23.9-fold after differentiation. The levels of the CGβ mRNA, on the other hand, increased only about 2-fold after differentiation for 2 weeks. Minor subpopulations of R278.5 cells differentiated even in the presence of fibroblasts, and the low level of α-fetoprotein, CGα, and CGβ mRNA present prior to the removal from fibroblasts could have been from these cells.

All SCID mice injected with R278.5 cells in either intramuscular or intratesticular sites formed tumors, and tumors in both sites demonstrated a similar range of differentiation. The

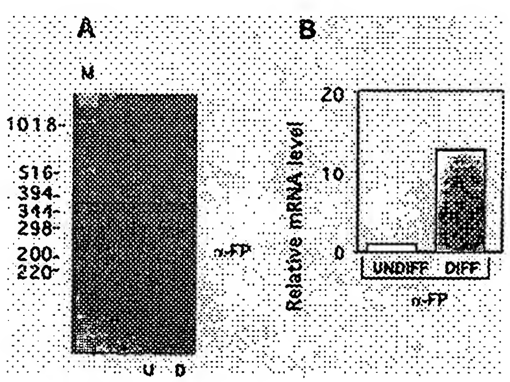

FIG. 3. Expression of α-fetoprotein mRNA. (*A*) PCR amplification of α-fetoprotein (αFP) cDNA from reverse-transcribed total RNA from undifferentiated (U) and differentiated (D) R278.5 cells. The DNA size markers (M) are indicated in bp. (*B*) The α-fetoprotein mRNA levels are expressed relative to the levels of the mRNA for G3PDH in each sample (not shown) as described in the text. Similar results were obtained in a second independent differentiation experiment.

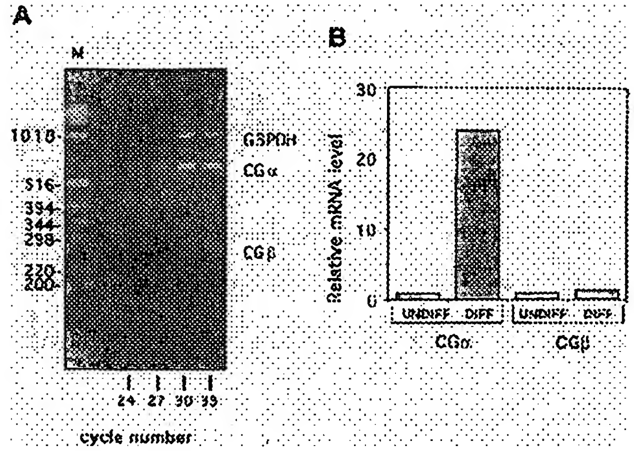

FIG. 4. Expression of CG subunit mRNA. (*A*) PCR amplification of cDNAs for G3PDH, CGα, and CGβ subunits from reverse-transcribed total RNA from differentiated R278.5 cells. The DNA size markers (M) are indicated in bp. (*B*) Relative levels of CGα and CGβ mRNAs in undifferentiated and differentiated R278.5 cells. Total RNA from cultured cells was analyzed for CG mRNA levels by RT-PCR and expressed relative to the levels of G3PDH mRNA. Similar results were obtained in a second independent differentiation experiment.

oldest tumors examined (15 weeks) had the most advanced differentiation, and all had abundant, unambiguous derivatives of all three embryonic germ layers, including ciliated columnar epithelium and nonciliated columnar epithelium (probable respiratory and gut epithelium; endoderm); bone, cartilage, smooth muscle, striated muscle (mesoderm); ganglia, other neural tissue, and stratified squamous epithelium (ectoderm), and other unidentified cell types (Fig. 5). Neural tissue included stratified cellular structures with remarkable resemblance to developing neural tube (Fig. 5*D*). Gut-like structures were often encircled by multiple layers of smooth muscle and were sometimes lined by villi with columnar epithelium interspersed with scattered mucus-secreting goblet cells (Fig. 5 *A* and *F*). Stratified squamous epithelium often contained well-differentiated hair follicles with hair shafts (Fig. 5*C*).

DISCUSSION

To our knowledge, there have been no previous reports of the isolation of a primate ES cell line. The characteristics that define R278.5 cells as ES cells include indefinite (>1 year) undifferentiated proliferation *in vitro*, maintenance of a normal karyotype, and potential to differentiate to derivatives of trophectoderm and all three embryonic germ layers. The development of complex structures in tumors in SCID mice with remarkable resemblance to normal hair follicles, neural tube, and gut demonstrates the ability of R278.5 cells to participate in complex developmental processes requiring coordinated interactions between multiple cell types. In the mouse embryo, the last cells capable of contributing to derivatives of trophectoderm and ICM are early ICM cells of the expanding blastocyst (29). The timing of commitment to ICM or trophectoderm has not been established for any primate species, but the potential of R278.5 cells to contribute to derivatives of both suggests that they most closely resemble early totipotent embryonic cells. The very limited ability of mouse ES cells to contribute to trophoblast in chimeras (30) suggests that the R278.5 cells represent an earlier developmental stage than mouse ES cells or that the ability of ICM cells to form trophectoderm persists longer in primates. Human EC cells share the ability of R278.5 cells to differentiate to trophoblast *in vitro* (16) and this potential may be a general distinguishing property of primate ES cell lines.

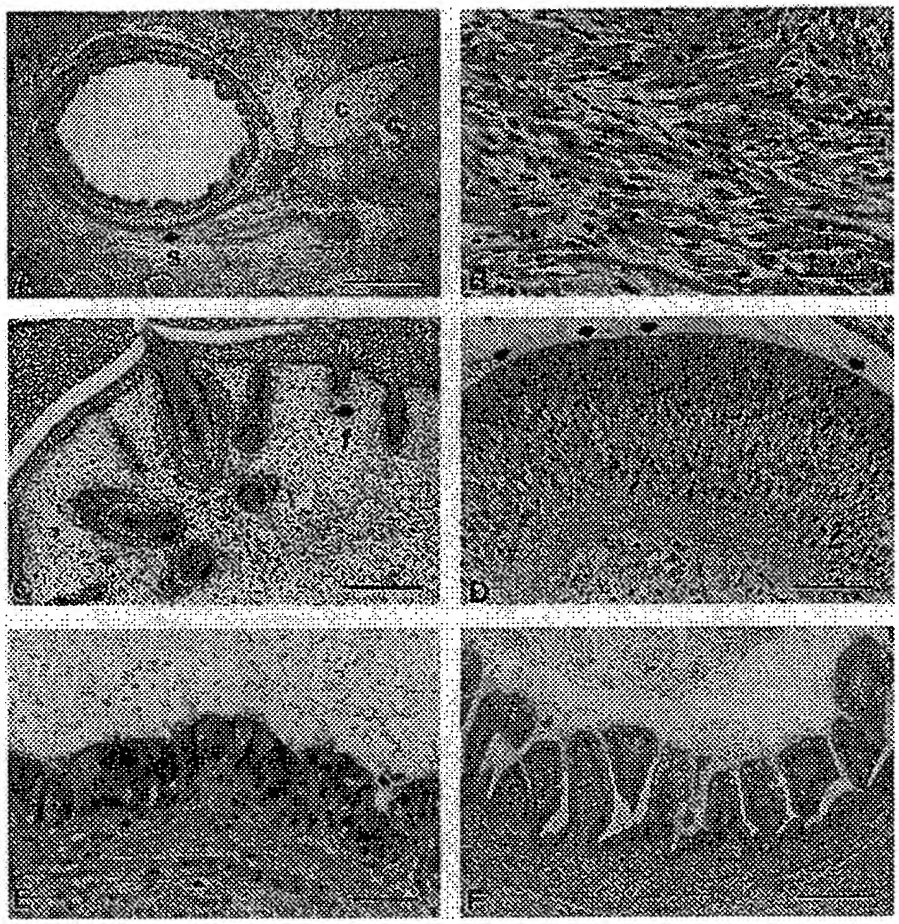

FIG. 5. Tumors formed by R278.5 cells injected into SCID mice and examined at 15 weeks. (*A*) Low-power field demonstrating disorganized differentiation of multiple cell types. A gut-like structure is encircled by smooth muscle (s), and elsewhere foci of cartilage (c) are present. (Bar = 400 μm.) (*B*) Striated muscle. (Bar = 40 μm.). (*C*) Stratified squamous epithelium with several hair follicles. The labeled hair follicle (f) has a visible hair shaft. (Bar = 200 μm.). (*D*) Stratified layers of neural cells in the pattern of a developing neural tube. An upper "ventricular" layer, containing numerous mitotic figures (arrows), overlies a lower "mantle" layer. (Bar = 100 μm.) (*E*) Ciliated columnar epithelium. (Bar = 40 μm.) (*F*) Villi covered with columnar epithelium with interspersed mucus-secreting goblet cells. (Bar = 200 μm.)

The only cells known to express the combination of markers alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, other than R278.5 cells are human EC cells (16, 25, 31). This expression pattern contrasts with undifferentiated mouse ES and EC cells, which instead express SSEA-1 and do not express SSEA-3, SSEA-4, TRA-1-60, or TRA-1-81 (23, 24). Differentiation of human EC cells such as NTERA2 cl.D1 (17) results in the loss of SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 expression and an increased SSEA-1 expression (16). These antigens have yet to be studied in early human or nonhuman primate embryos, and their functions are unknown, but their shared expression by R278.5 cells and human EC cells suggests a close embryological similarity.

In the absence of fibroblast feeder layers, soluble LIF fails to prevent the differentiation of R278.5 cells or of feeder-dependent human EC cells (19). The factors that fibroblasts produce that prevent the differentiation of R278.5 cells or feeder-dependent human EC cells are unknown. Other factors that fail to support the growth of feeder-dependent human EC cells in the absence of feeder layers include oncostatin M and ciliary neurotrophic factor (19), both of which can substitute for LIF in preventing the differentiation of mouse ES cells (32, 33). A trypsin-sensitive factor from a human yolk sac carcinoma cell line (GCT 44) supports the growth of feeder-dependent human EC cells in the absence of fibroblasts, but the factor has not yet been purified (19).

Although exogenous LIF was added during the initial derivation of R278.5 cells, the cell line is now routinely passaged without added LIF. We have also recently derived two additional cell lines (R366 and R367) from four additional rhesus blastocysts, using the same techniques as described for R278.5 cells, but without added LIF (data not shown). R366 and R367 cells have normal karyotypes and continue to proliferate *in vitro* for at least 3 months. R366 and R367 cell lines have not yet been tested for tumor formation in SCID mice, but they are indistinguishable from R278.5 cells in undifferentiated morphology, growth characteristics, and *in vitro* differentiation in the absence of feeder layers.

The differentiation of R278.5 cells to trophoblast was demonstrated by the expression of CGα and CGβ subunit mRNAs and the secretion of bioactive CG into the culture medium by differentiated ES cells. We were surprised to note that while the relative levels of the CGα subunit were increased >20 times in differentiated cells, the relative levels of the CGβ subunit only changed about 2-fold. The fact that CG secretion increased substantially with differentiation may mean that under our *in vitro* culture conditions, expression of the CGα subunit is limiting for CG secretion. CGβ subunit mRNA is detectable in human preimplantation embryos as early as the eight-cell stage, which is before trophectoderm differentiation (34), consistent with a low level of CGβ mRNA expression in undifferentiated R278.5 cells. Although there may be some coordinate mechanisms regulating CGα and CGβ gene transcription in the placenta (35), it is clear that there is differential regulation of these genes *in vitro* and *in vivo* (36). Since the expression of the CGβ subunit is also divergent among villous

and extravillous trophoblasts (37), further studies are needed to determine the phenotype of the trophoblasts derived from R278.5 cells.

Primate ES cells will be particularly useful for *in vitro* developmental studies of lineages that differ substantially between humans and mice. However, the most accurate *in vitro* model of the differentiation of human tissues would be provided by human ES cells. In one published report, ICM-derived cells from spare *in vitro* fertilized human embryos were cultured with LIF in the absence of feeder layers, and, although alkaline phosphatase positive cells proliferated, they failed to survive beyond two passages (38). These results suggest that soluble LIF alone will not prevent the differentiation of human ES cells, just as it fails to prevent the differentiation of rhesus ES cells. The growth of rhesus monkey ES cells in culture conditions that support feeder-dependent human EC cells suggests that similar conditions may support human ES cells.

Human ES cells would offer exciting new possibilities for transplantation medicine. Because ES cells have the developmental potential to give rise to all adult cell types, any disease resulting from the failure of specific cell types would be potentially treatable through the transplantation of differentiated cells derived from ES cells. Because ES cells are immortal cell lines, they could be genetically manipulated prior to differentiation either to reduce immunogenicity or to give them new properties to combat specific diseases. Rhesus monkey ES cells and rhesus monkeys will be invaluable for testing the safety and efficacy of the transplantation of specific cell types for the treatment of specific diseases. Because of the range of diseases potentially treatable by this approach, elucidating the basic mechanisms controlling the differentiation of primate ES cells has dramatic clinical significance.

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1. Martin, G. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638.
2. Evans, M. & Kaufman, M. (1981) *Nature (London)* **292**, 154–156.
3. Matsui, Y., Zsebo, K. & Hogan, B. L. M. (1992) *Cell* **70**, 841–847.
4. Resnick, J. L., Bixler, L. S., Cheng, L. & Donovan, P. J. (1992) *Nature (London)* **359**, 550–551.
5. Doetschman, T., Williams, P. & Maeda, N. (1988) *Dev. Biol.* **127**, 224–227.
6. Iannaccone, P. M., Taborn, G. U., Garton, R. L., Caplice, M. D. & Brenin, D. R. (1994) *Dev. Biol.* **163**, 288–292.
7. Evans, M., Notaranni, E., Laurie, S. & Moor, R. (1990) *Theriogenology* **33**, 125–128.
8. Graves, K. H. & Moreadith, R. W. (1993) *Mol. Reprod. Dev.* **36**, 424–433.
9. Notarianni, E., Galli, C., Laurie, S., Moore, R. M. & Evans, M. J. (1991) *J. Reprod. Fertil. Suppl.* **43**, 255–260.
10. Sukoyan, M. A., Golubitsa, A. N., Zhelezova, A. I., Shilov, A. G., Vatolin, S. Y., Maximovsky, L. P., Andreeva, L. E., McWhir, J., Pack, S. D., Bayborodin, S. I., Kerkis, A. Y., Kizilova, H. I. & Serov, O. L. (1992) *Mol. Reprod. Dev.* **33**, 418–431.
11. Williams, R., Hilton, D., Pease, S., Wilson, T., Stewart, C., Gearing, D., Wagner, E., Metcalf, D., Nicola, N. & Gough, N. (1988) *Nature (London)* **336**, 684–687.
12. Bradley, A., Evans, M., Kaufman, M. & Robertson, E. (1984) *Nature (London)* **309**, 255–256.
13. Ramirez Solis, R., Davis, A. C. & Bradley, A. (1993) *Methods Enzymol.* **225**, 855–878.
14. Braude, P., Bolton, V. & Moore, S. (1988) *Nature (London)* **332**, 459–461.
15. Benirschke, K. & Kaufmann, P. (1990) *Pathology of the Human Placenta* (Springer, New York).
16. Andrews, P., Oosterhuis, J. & Damjanov, I. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, ed. Robertson, E. (IRL, Oxford), pp. 207–246.
17. Andrews, P., Damjanov, I., Simon, D., Banting, G., Carlin, C., Dracopoli, N. & Fogh, J. (1984) *Lab. Invest.* **50**, 147–162.
18. Pera, M. F., Blasco Lafita, M. J. & Mills, J. (1987) *Int. J. Cancer* **40**, 334–343.
19. Roach, S., Cooper, S., Bennett, W. & Pera, M. F. (1993) *Eur. Urol.* **23**, 82–88.
20. Seshagiri, P. B., Bridson, W. E., Dierschke, D. J., Eisele, S. G. & Hearn, J. P. (1993) *Am. J. Primatol.* **29**, 81–91.
21. Solter, D. & Knowles, B. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 5099–5102.
22. Robertson, E. J. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. ed. Robertson, E. J. (IRL, Oxford), pp. 71–112.
23. Solter, D. & Knowles, B. B. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5565–5569.
24. Kannagi, R., Cochran, N. A., Ishigami, F., Hakomori, S., Andrews, P. W., Knowles, B. B. & Solter, D. (1983) *EMBO J.* **2**, 2355–2361.
25. Andrews, P. W., Banting, G., Damjanov, I., Arnaud, D. & Avner, P. (1984) *Hybridoma* **3**, 347–361.
26. Terasawa, E., Bridson, W. E., Nass, T. E., Noonan, J. J. & Dierschke, D. J. (1984) *Endocrinology* **115**, 2233–2240.
27. Golos, T. G., Durning, M., Fisher, J. M. & Fowler, P. D. (1993) *Endocrinology* **133**, 1744–1752.
28. Golos, T. G., Durning, M. & Fisher, J. M. (1991) *DNA Cell Biol.* **10**, 367–379.
29. Winkel, G. K. & Pedersen, R. A. (1988) *Dev. Biol.* **127**, 143–156.
30. Beddington, R. S. P. & Robertson, E. J. (1989) *Development (Cambridge, U.K.)* **105**, 733–737.
31. Wenk, J., Andrews, P. W., Casper, J., Hata, J., Pera, M. F., von Keitz, A., Damjanov, I. & Fenderson, B. A. (1994) *Int. J. Cancer* **58**, 108–115.
32. Wolf, E., Kramer, R., Polejaeva, I., Thoenen, H. & Brem, G. (1994) *Transgenic Res.* **3**, 152–158.
33. Rose, T. M., Wieford, D. M., Gunderson, N. L. & Bruce, A. G. (1994) *Cytokine* **6**, 48–54.
34. Bonduelle, M. L., Dodd, R., Liebaers, I., Van Steirteghem, A., Williamson, R. & Akhurst, R. (1988) *Hum. Reprod.* **3**, 909–914.
35. Steger, D. J., Buscher, M., Hecht, J. H. & Mellon, P. L. (1993) *Mol. Endocrinol.* **7**, 1579–1588.
36. Jameson, J. L. & Hollenberg, A. N. (1993) *Endocr. Rev.* **14**, 203–221.
37. Babury, R. A. & Moscovic, E. A. (1993) *Histol. Histopathol.* **8**, 323–328.
38. Bongso, A., Fong, C. Y., Ng, S. C. & Ratnam, S. (1994) *Hum. Reprod.* **9**, 2110–2117.